

08/29/97

A #

Patent  
Attorney's Docket No. 012712-432

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

UTILITY PATENT  
APPLICATION TRANSMITTAL LETTER

**Box PATENT APPLICATION**  
Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Enclosed for filing is the utility patent application of Darrell R. ANDERSON; Nabil HANNA; John E. LEONARD; Roland A. NEWMAN; Mitchell E. REFF; and William H. RASTETTER for THERAPEUTIC APPLICATION OF CHIMERIC AND RADIOLABELED ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED DIFFERENTIATION ANTIGEN FOR TREATMENT OF B CELL LYMPHOMA.

Also enclosed are:

- ☒ Twenty-one (21) sheets of ☒ formal ☐ informal drawings;
- ☐ a claim for foreign priority under 35 U.S.C. §§ 119 and/or 365 in ☐ a separate document ☐ the declaration;
- ☐ a certified copy of the priority document;
- ☐ an Associate Power of Attorney;
- ☒ One unexecuted verified statement claiming small entity status;
- ☐ an Assignment document;
- ☒ a Request for Listing of References and three (3) Form 1449s; and
- ☒ Other: Information Sheet; and Preliminary Amendment

The declaration of the inventors ☐ also is enclosed ☒ will follow.

The filing fee has been calculated as follows ☒ and in accordance with the enclosed preliminary amendment:

C L A I M S					
	NO. OF CLAIMS		EXTRA CLAIMS	RATE	FEE
Basic Application Fee					\$ 770.00
Total Claims	10	MINUS 20 =	0	x \$22 =	
Independent Claims	1	MINUS 3 =	0	x \$80 =	
If multiple dependent claims are presented, add \$260.00					
Total Application Fee					770.00

If verified statement claiming small entity status is enclosed, subtract 50% of Total Application Fee	385.00
Add Assignment Recording Fee of \$40.00 if Assignment document is enclosed	
<b>TOTAL APPLICATION FEE DUE</b>	<b>\$385.00</b>

[X] A check in the amount of \$ 385.00 is enclosed for the fee due.

[ ] Charge \$ \_\_\_\_\_ to Deposit Account No. 02-4800 for the fee due.

The Commissioner is hereby authorized to charge any appropriate fees under 37 C.F.R. §§ 1.16, 1.17 and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 02-4800. This paper is submitted in triplicate.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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Date: August 29, 1997

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of )  
 )  
Darrell R. ANDERSON et al. ) Group Art Unit: Unassigned  
 )  
Application No.: Unassigned ) Examiner: Unassigned  
(CIP of Serial No. 08/149,099) )  
 )  
Filed: August 29, 1997 )  
 )  
For: THERAPEUTIC APPLICATION OF )  
CHIMERIC AND RADIOLABELED )  
ANTIBODIES TO HUMAN B )  
LYMPHOCYTE RESTRICTED... )

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination, kindly amend the above-identified  
application as follows:

**IN THE SPECIFICATION**

In compliance with 37 C.F.R. § 1.823(a), please insert the  
attached paper copy of the "Sequence Listing" between the last  
page of the Disclosure (page 62) and the first page of the  
Claims to replace the Sequence Listing previously filed in the  
parent application on December 8, 1994.

Page 5, line 8, after "ie", add a --,-- symbol;  
line 22, after "ie", add a --,-- symbol;  
line 27, after --eg--, add a --,-- symbol.

Page 6, line 17, after "ie", add a --,-- symbol.

Page 7, lines 12 and 13, after "ie", add a --,-- symbol.

Page 8, line 14, after "ie", add the --,-- symbol;  
line 22, after "eg", add a --,-- symbol.

Page 9, line 25, after "sequence" insert --(SEQ. ID. NO.  
2) --;  
line 27, after "sequence" insert --(SEQ. ID. NO.  
3) --.

Page 10, line 1, after "sequences" insert --(SEQ. ID. NO.  
6) --;  
line 6, after "sequences" insert --(SEQ. ID. NO.  
9) --.

Page 14, line 26, change "parenterally administerable" to  
--parenterally administrable--.

Page 15, line 6, after "ie", add a --,-- symbol;  
line 20, after "ie", add a --,-- symbol.

Page 16, line 20, after "ie", add a --,-- symbol.

Page 17, lines 5 and 11, after "eg", add a --,-- symbol;  
line 5, change "furthermore" to read --Addition-  
ally--.



Page 18, lines 4 and 10, after "ie", add a --,-- symbol;  
line 10, after "ie", add a --,-- symbol;  
lines 17 and 26 and 28, after "eg", add a --,--  
symbol.

Page 19, line 11, after "88/04936" add a --,-- symbol.

Page 20, line 13, after "eg", add a --,-- symbol.

Page 21, line 3, after "ie", add a --,-- symbol.

Page 22, line 17, after "sequence", insert --(SEQ. ID. NO.  
1)--;  
line 27, amend "(SEQ. ID. NO. 1)" to read --(SEQ.  
ID. NO. 2)--.

Page 23, line 29, amend "(SEQ. ID. NO. 2)" to read --(SEQ.  
ID. NO. 3)--.

Page 26, line 18, after "ie", add a --,-- symbol;  
line 20, change "washed with Alconox and rinsed  
with MILLI-Q water" to the following: --washed with an appro-  
priate detergent compound (e.g., ALCONOX®) and rinsed with  
purified water (e.g., water purified by the MILLI-Q®  
apparatus)--;

line 20, delete the following text (introduced in

the amendment submitted on November 7, 1996): "washed with an appropriate detergent compound (e.g., ALCONOX®) and rinsed with purified water (e.g., water purified by the MILLI-Q® apparatus)", and substitute the following text:

--washed with ALCONOX® (a detergent) and rinsed with MILLI-Q water (purified water)--.

line 23, change "Milli-Q" to --MILLI-Q®--.

Page 37, line 26, after "ie", add a --,-- symbol.

Page 40, line 24, amend "(SEQ. ID. NO. 3)" to --(SEQ. ID. NO. 4)--;

last line, amend "(SEQ. ID. NO. 4)" to --(SEQ. ID. NO. 5)--.

Page 41, line 6, after "2", insert --(SEQ. ID. NO. 2)--;  
line 7, after "3", insert --(SEQ. ID. NO. 3)--;  
line 12, amend "(SEQ. ID. NO. 5)" to read --(SEQ. ID. NO. 6)--; and after "3", insert --(SEQ. ID. NO. 3)--;

line 13, after "4", insert --(SEQ. ID. NO. 6)--;  
line 20, amend "(SEQ. ID. NO. 6)" to read --(SEQ. ID. NO. 7)--;

line 26, amend "(SEQ. ID. NO. 7)" to read --(SEQ. ID. NO. 8)--.

Page 42, line 4, after "2", insert --(SEQ. ID. NO. 9)--;

line 8, after "3", insert --(SEQ. ID. NO. 3)--.

Page 43, line 22, after "ie", add a --,-- symbol.

Page 44, line 23, after "ie", add a --,-- symbol.

Page 61, line 6, after "eg", each occurrence, add --,--  
symbol.

Page 62, line 10, after "(ATCC)" and before the comma  
", "insert the following: --on November 4, 1992--.

REMARKS

Entry of the foregoing amendments and favorable considera-  
tion of the subject application, as amended, is respectfully  
requested.

Respectfully submitted,

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Robin L. Teskin  
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Date: August 29, 1997

**THERAPEUTIC APPLICATION OF CHIMERIC AND RADIOLABELED  
ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED DIFFERENTIATION  
ANTIGEN FOR TREATMENT OF B CELL LYMPHOMA**

**37 C.F.R. §1.74(d)/(e) Copyright Notice**

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right owner does not object to the reproduction by anyone of the patent document or the patent disclosure, as it appears in the Patent and Trademark Office patent files or records, but otherwise reserves all copyright rights whatsoever.

**RELATED APPLICATIONS**

This application is a continuation-in-part of U.S. Serial No. 08/149,099, filed November 3, 1993, in turn a continuation-in-part of U.S. Serial No. 07/978,891, filed November 13, 1992, and now abandoned. This patent document is related to U.S. Serial No. 07/9077,691, entitled "Impaired Dominant Selectable Marker Sequene for Enhancement of Expression of Co-Linked Gene Product and Expression Vector Systems Comprising Same", filed November 13, 1992, now abandoned, and U.S. Serial No. 08/147,696, entitled "Impaired Dominant Selectable Marker Sequence and Intronic Insertion Strategies for Enhancement of Expression of Gene Product and Expression Vector Systems Comprising Same", filed November 3, 1993, now U.S. Patent 5,648,267. The related patent documents are incorporated herein by reference.

## TABLE OF CONTENTS

	A.	FIELD OF THE INVENTION
	B.	BACKGROUND OF THE INVENTION
5	C.	SUMMARY OF THE INVENTION
	D.	BRIEF DESCRIPTION OF THE DRAWINGS
	E.	DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS
	F.	EXAMPLES
	I.	RADIOLABELED ANTI-CD20 ANTIBODY 2B8
10	A.	Anti-CD20 Monoclonal Antibody (Murine) Production ("2B8")
	B.	Preparation of 2B8-MX-DTPA Conjugate
	i.	MX-DTPA
	ii.	Preparation of 2B8
	iii.	Conjugation of 2B8 with MX-DTPA
15	iv.	Determination of MX-DTPA Incorporation
	v.	Immunoreactivity of 2B8-MX-DTPA
	vi.	Preparation of Indium-[111]-labeled 2B8-MX-DTPA ("I2B8")
	vii.	Preparation of Yttrium-[90]-labeled 2B8-MX-DTPA ("Y2B8")
20	C.	Non-Human Animal Studies
	i.	Distribution of Radiolabeled 2B8-MX-DTPA
	ii.	Tumor Localization of I2B8
25	iii.	Biodistribution and Tumor Localization Studies with Radiolabeled 2B8-MX-DTPA
	D.	Human Studies
	i.	2B8 and 2B8-MX-DTPA: Immunohistology Studies with Human Tissues
30	ii.	Clinical Analysis of I2B8 (Imaging) and Y2B8 (Therapy)
	a.	Phase I/II Clinical Trial: Single Dose Therapy Study
35	b.	Phase I/II Clinical Trial: Multiple Dose Therapy Study

## II. CHIMERIC ANTI-CD20 PRODUCTION

A. Construction of Chimeric Anti-CD20 Immunoglobulin DNA Expression Vectors

B. Creation of Chimeric Anti-CD20 Producing CHO and SP2/0 Transfectomas

C. Determination of Immunological Activity of Chimeric Anti-CD20 Antibodies

i. Human Clq Analysis

ii. Complement Dependent Cell Lyses

iii. Antibody Dependent Cellular Cytotoxicity Effector Assay

## III. DEPLETION OF B CELLS IN VIVO USING CHIMERIC ANTI-CD20

A. Non-Human Primate Study

B. Clinical Analysis of C2B8

i. Phase I/II Clinical Trial of C2B8: Single Dose Therapy Study

ii. Phase I/II Clinical Trial of C2B8: Multiple Dose Therapy Study

## IV. COMBINATION THERAPY: C2B8 AND Y2B8

A. Preparation of Y2B8

B. Preparation of C2B8

C. Results

## V. ALTERNATIVE THERAPY STRATEGIES

## VI. DEPOSIT INFORMATION

VII. IN VIVO (HUMAN) Y2B8 DOSIMETRY STUDIES

VIII. IN VIVO (HUMAN) Y2B8/C2B8 COMBINATION RADIOTHERAPY

SEQUENCE LISTING  
CLAIMS

### A. FIELD OF THE INVENTION

The references to be discussed throughout this document are set forth merely for the information described therein prior to the filing dates of this document, and  
5 nothing herein is to be construed as an admission, either express or implied, that the references are "prior art" or that the inventors are not entitled to antedate such descriptions by virtue of prior inventions or priority based on earlier filed applications.

- 10 The present invention is directed to the treatment of B cell lymphoma using chimeric and radiolabeled antibodies to the B cell surface antigen Bp35 ("CD20").

### B. BACKGROUND OF THE INVENTION

- 15 The immune system of vertebrates (for example, primates, which include humans, apes, monkeys, etc.) consists of a number of organs and cell types which have evolved to: accurately and specifically recognize foreign microorganisms ("antigen") which invade the vertebrate-host; specifically bind to such foreign microorganisms; and, eliminate/destroy such foreign microorganisms.
- 20 Lymphocytes, amongst others, are critical to the immune system. Lymphocytes are produced in the thymus, spleen and bone marrow (adult) and represent about 30% of the total white blood cells present in the circulatory system of humans (adult). There are two major sub-populations of lymphocytes: T cells and B cells. T cells are responsible for cell mediated immunity, while B cells are responsible  
25 for antibody production (humoral immunity). However, T cells and B cells can be considered as interdependent--in a typical immune response, T cells are activated when the T cell receptor binds to fragments of an antigen that are bound to major histocompatibility complex ("MHC") glycoproteins on the surface of an antigen presenting cell; such activation causes release of biological

mediators ("interleukins") which, in essence, stimulate B cells to differentiate and produce antibody ("immunoglobulins") against the antigen.

Each B cell within the host expresses a different antibody on its surface--thus,  
5 one B cell will express antibody specific for one antigen, while another B cell will express antibody specific for a different antigen. Accordingly, B cells are quite diverse, and this diversity is critical to the immune system. In humans, each B cell can produce an enormous number of antibody molecules (*ie* about  $10^7$  to  $10^8$ ). Such antibody production most typically ceases (or substantially decreases) when  
10 the foreign antigen has been neutralized. Occasionally, however, proliferation of a particular B cell will continue unabated; such proliferation can result in a cancer referred to as "B cell lymphoma."

T cells and B cells both comprise cell surface proteins which can be utilized as  
15 "markers" for differentiation and identification. One such human B cell marker is the human B lymphocyte-restricted differentiation antigen Bp35, referred to as "CD20." CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation. Specifically, the CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and  
20 differentiation and is usually expressed at very high levels on neoplastic ("tumor") B cells. CD20, by definition, is present on both "normal" B cells as well as "malignant" B cells, *ie* those B cells whose unabated proliferation can lead to B cell lymphoma. Thus, the CD20 surface antigen has the potential of serving as a candidate for "targeting" of B cell lymphomas.

25 In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B cells are, *eg* injected into a patient. These anti-CD20 antibodies specifically bind to the CD20 cell surface antigen of (ostensibly) both normal and malignant B cells; the anti-CD20 antibody bound to the CD20



surface antigen may lead to the destruction and depletion of neoplastic B cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is specifically "delivered" to, *eg.* the neoplastic B cells. Irrespective of the approach, a primary goal is to destroy the tumor: the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably.

For example, attempts at such targeting of CD20 surface antigen have been reported. Murine (mouse) monoclonal antibody 1F5 (an anti-CD20 antibody) was reportedly administered by continuous intravenous infusion to B cell lymphoma patients. Extremely high levels (>2 grams) of 1F5 were reportedly required to deplete circulating tumor cells, and the results were described as being "transient." Press *et al.*, "Monoclonal Antibody 1F5 (Anti-CD20) Serotherapy of Human B-Cell Lymphomas." *Blood* 69/2:584-591 (1987). A potential problem with this approach is that non-human monoclonal antibodies (*eg.* murine monoclonal antibodies) typically lack human effector functionality, *ie* they are unable to, *inter alia*, mediate complement dependent lysis or lyse human target cells through antibody dependent cellular toxicity or Fc-receptor mediated phagocytosis. Furthermore, non-human monoclonal antibodies can be recognized by the human host as a foreign protein; therefore, repeated injections of such foreign antibodies can lead to the induction of immune responses leading to harmful hypersensitivity reactions. For murine-based monoclonal antibodies, this is often referred to as a Human Anti-Mouse Antibody response, or "HAMA" response. Additionally, these "foreign" antibodies can be attacked by the immune system of the host such that they are, in effect, neutralized before they reach their target site.



Monoclonal Antibodies in the Treatment of Non-Hodgkin's Lymphoma"

*Hem./Onc. Clinics of N.A.* 5/5:1013-1025 (1991) (review article); Press, O.W. *et al* "Radiolabeled-Antibody Therapy of B-Cell Lymphoma with Autologous Bone Marrow Support." *New England Journal of Medicine* 329/17: 1219-12223

- 5 (1993) (iodine-131 labeled anti-CD20 antibody IF5 and B1); and Kaminski, M.G. *et al* "Radioimmunotherapy of B-Cell Lymphoma with [<sup>131</sup>I] Anti-B1 (Anti-CD20) Antibody". *NEJM* 329/7 (1993) (iodine-131 labeled anti-CD20 antibody B1; hereinafter "Kaminski").

- 10 Toxins (*ie* chemotherapeutic agents such as doxorubicin or mitomycin C) have also been conjugated to antibodies. *See, for example*, PCT published application WO 92/07466 (published May 14, 1992).

- 15 "Chimeric" antibodies, *ie* antibodies which comprise portions from two or more different species (*eg*, mouse and human) have been developed as an alternative to "conjugated" antibodies. For example, Liu, A.Y. *et al.*, "Production of a Mouse-Human Chimeric Monoclonal Antibody to CD20 with Potent Fc-Dependent Biologic Activity" *J. Immun.* 139/10:3521-3526 (1987), describes a mouse/human chimeric antibody directed against the CD20 antigen. *See also*, PCT Publication
- 20 No. WO 88/04936. However, no information is provided as to the ability, efficacy or practicality of using such chimeric antibodies for the treatment of B cell disorders in the reference. It is noted that *in vitro* functional assays (*eg* complement dependent lysis ("CDC"); antibody dependent cellular cytotoxicity ("ADCC"), etc.) cannot inherently predict the *in vivo* capability of a chimeric
- 25 antibody to destroy or deplete target cells expressing the specific antigen. *See, for example*, Robinson, R.D. *et al.*, "Chimeric mouse-human anti-carcinoma antibodies that mediate different anti-tumor cell biological activities," *Hum. Antibod. Hybridomas* 2:84-93 (1991) (chimeric mouse-human antibody having

undetectable ADCC activity). Therefore, the potential therapeutic efficacy of chimeric antibody can only truly be assessed by *in vivo* experimentation.

What is needed, and what would be a great advance in the art, are therapeutic approaches targeting the CD20 antigen for the treatment of B cell lymphomas in primates, including, but not limited to, humans.

### C. SUMMARY OF THE INVENTION

Disclosed herein are therapeutic methods designed for the treatment of B cell disorders, and in particular, B cell lymphomas. These protocols are based upon the administration of immunologically active chimeric anti-CD20 antibodies for the depletion of peripheral blood B cells, including B cells associated with lymphoma; administration of radiolabeled anti-CD20 antibodies for targeting localized and peripheral B cell associated tumors; and administration of chimeric anti-CD20 antibodies and radiolabeled anti-CD20 antibodies in a cooperative therapeutic strategy.

### D. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of a tandem chimeric antibody expression vector useful in the production of immunologically active chimeric anti-CD20 antibodies ("TCAE 8");

Figures 2A through 2E are the nucleic acid sequence of the vector of Figure 1;

Figures 3A through 3F are the nucleic acid sequence of the vector of Figure 1 further comprising murine light and heavy chain variable regions ("anti-CD20 in TCAE 8");

Figure 4 is the nucleic acid and amino acid sequences (including CDR and framework regions) of murine variable region light chain derived from murine anti-CD20 monoclonal antibody 2B8;

5

Figure 5 is the nucleic acid and amino acid sequences (including CDR and framework regions) of murine variable region heavy chain derived from murine anti-CD20 monoclonal antibody 2B8;

10 Figure 6 are flow cytometry results evidencing binding of fluorescent-labeled human C1q to chimeric anti-CD20 antibody, including, as controls labeled C1q; labeled C1q and murine anti-CD20 monoclonal antibody 2B8; and labeled C1q and human IgG1,k;

15 Figure 7 represents the results of complement related lysis comparing chimeric anti-CD20 antibody and murine anti-CD20 monoclonal antibody 2B8;

Figure 8 represents the results of antibody mediated cellular cytotoxicity with *in vivo* human effector cells comparing chimeric anti-CD20 antibody and 2B8;

20

Figure 9A, 9B and 9C provide the results of non-human primate peripheral blood B lymphocyte depletion after infusion of 0.4 mg/kg (A); 1.6 mg/kg (B); and 6.4 mg/kg (C) of immunologically active chimeric anti-CD20 antibody;

25 Figure 10 provides the results of, *inter alia*, non-human primate peripheral blood B lymphocyte depletion after infusion of 0.01 mg/kg of immunologically active chimeric anti-CD20 antibody;

Figure 11 provides results of the tumoricidal impact of Y2B8 in a mouse xenographic model utilizing a B cell lymphoblastic tumor;

Figure 12 provides results of the tumoricidal impact of C2B8 in a mouse  
5 xenographic model utilizing a B cell lymphoblastic tumor;

Figure 13 provides results of the tumoricidal impact of a combination of Y2B8 and C2B8 in a mouse xenographic model utilizing a B cell lymphoblastic tumor; and

10 Figures 14A and 14B provide results from a Phase I/II clinical analysis of C2B8 evidencing B-cell population depletion over time for patients evidencing a partial remission of the disease (14A) and a minor remission of the disease (14B).

#### **E. DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

15 Generally, antibodies are composed of two light chains and two heavy chain molecules; these chains form a general "Y" shape, with both light and heavy chains forming the arms of the Y and the heavy chains forming the base of the Y. Light and heavy chains are divided into domains of structural and functional  
20 homology. The variable domains of both the light ("V<sub>L</sub>") and the heavy ("V<sub>H</sub>") chains determine recognition and specificity. The constant region domains of light ("C<sub>L</sub>") and heavy ("C<sub>H</sub>") chains confer important biological properties, eg antibody chain association, secretion, transplacental mobility, Fc receptor binding complement binding, etc. The series of events leading to  
25 immunoglobulin gene expression in the antibody producing cells are complex. The variable domain region gene sequences are located in separate germ line gene segments referred to as "V<sub>H</sub>," "D," and "J<sub>H</sub>," or "V<sub>L</sub>" and "J<sub>L</sub>." These gene segments are joined by DNA rearrangements to form the complete V regions expressed in heavy and light chains, respectively. The rearranged, joined V

segments ( $V_L-J_L$  and  $V_H-D-J_H$ ) then encode the complete variable regions or antigen binding domains of light and heavy chains, respectively.

Serotherapy of human B cell lymphomas using an anti-CD20 murine monoclonal antibody (1F5) has been described by Press *et al.*, (69 *Blood* 584, 1987, *supra*);  
5 the reported therapeutic responses, unfortunately, were transient. Additionally, 25% of the tested patients reportedly developed a human anti-mouse antibody (HAMA) response to the serotherapy. Press *et al.*, suggest that these antibodies, conjugated to toxins or radioisotopes, might afford a more lasting clinical benefit  
10 than the unconjugated antibody.

Owing to the debilitating effects of B cell lymphoma and the very real need to provide viable treatment approaches to this disease, we have embarked upon different approaches having a particular antibody, 2B8, as the common link  
15 between the approaches. One such approach advantageously exploits the ability of mammalian systems to readily and efficiently recover peripheral blood B cells; using this approach, we seek to, in essence, purge or deplete B cells in peripheral blood and lymphatic tissue as a means of also removing B cell lymphomas. We accomplish this by utilization of, *inter alia*, immunologically active, chimeric  
20 anti-CD20 antibodies. In another approach, we seek to target tumor cells for destruction with radioactive labels.

As used herein, the term "anti-CD20 antibody" is an antibody which specifically recognizes a cell surface non-glycosylated phosphoprotein of 35,000 Daltons,  
25 typically designated as the human B lymphocyte restricted differentiation antigen Bp35, commonly referred to as CD20. As used herein, the term "chimeric" when used in reference to anti-CD20 antibodies, encompasses antibodies which are most preferably derived using recombinant deoxyribonucleic acid techniques and which comprise both human (including

immunologically "related" species, eg, chimpanzee) and non-human components: the constant region of the chimeric antibody is most preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human source and has the desired antigenic and specificity to the CD20 cell surface antigen. The non-human source can be any vertebrate source which can be used to generate antibodies to a human CD20 cell surface antigen or material comprising a human CD20 cell surface antigen. Such non-human source includes, but is not limited to, rodents (eg, rabbit, rat, mouse, etc.) and non-human primates (eg, Old World Monkey, Ape, etc.). Most preferably, the non-human component (variable region) is derived from a murine source. As used herein, the phrase "immunologically active" when used in reference to chimeric anti-CD20 antibodies, means a chimeric antibody which binds human C1q, mediates complement dependent lysis ("CDC") of human B lymphoid cell lines, and lyses human target cells through antibody dependent cellular cytotoxicity ("ADCC"). As used herein, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to an antibody and at least one radionuclide is inserted into the chelating agent. Preferred chelating agents and radionuclides are set forth in Srivagtava, S.C. and Mease, R.C., "Progress in Research on Ligands, Nuclides and Techniques for Labeling Monoclonal Antibodies," *Nucl. Med. Bio.* 18/6: 589-603 (1991) ("Srivagtava") which is incorporated herein by reference. A particularly preferred chelating agent is 1-isothiocymatobenzyl-3-methyldiothelene triaminepent acetic acid ("MX-DTPA"); particularly preferred radionuclides for indirect labeling include indium [111] and yttrium [90]. As used herein, the phrases "direct labeling" and "direct labeling approach" both mean that a radionuclide is covalently attached directly to an antibody (typically via an amino acid residue). Preferred radionuclides are provided in Srivagtava; a particularly preferred radionuclide



for direct labeling is iodine [131] covalently attached via tyrosine residues. The indirect labeling approach is particularly preferred.

5 The therapeutic approaches disclosed herein are based upon the ability of the immune system of primates to rapidly recover, or rejuvenate, peripheral blood B cells. Additionally, because the principal immune response of primates is occasioned by T cells, when the immune system has a peripheral blood B cell deficiency, the need for "extraordinary" precautions (*ie* patient isolation, etc.) is not necessary. As a result of these and other nuances of the immune systems of  
10 primates, our therapeutic approach to B cell disorders allows for the purging of peripheral blood B cells using immunologically active chimeric anti-CD20 antibodies.

Because peripheral blood B cell disorders, by definition, can indicate a necessity  
15 for access to the blood for treatment, the route of administration of the immunologically active chimeric anti-CD20 antibodies and radioalabeled anti-CD20 antibodies is preferably parenteral; as used herein, the term "parenteral" includes intravenous, intramuscular, subcutaneous, rectal, vaginal or intraperitoneal administration. Of these, intravenous administration is most  
20 preferred.

The immunologically active chimeric anti-CD20 antibodies and radiolabeled anti-CD20 antibodies will typically be provided by standard technique within a pharmaceutically acceptable buffer, for example, sterile saline, sterile buffered  
25 water, propylene glycol, combinations of the foregoing, etc. Methods for preparing parenterally administerable agents are described in *Pharmaceutical Carriers & Formulations*, Martin, Remington's Pharmaceutical Sciences, 15th Ed. (Mack Pub. Co., Easton, PA 1975), which is incorporated herein by reference.

The specific, therapeutically effective amount of immunologically active chimeric anti-CD20 antibodies useful to produce a unique therapeutic effect in any given patient can be determined by standard techniques well known to those of ordinary skill in the art.

5

Effective dosages (*ie* therapeutically effective amounts) of the immunologically active chimeric anti-CD20 antibodies range from about 0.001 to about 30 mg/kg body weight, more preferably from about 0.01 to about 25 mg/kg body weight, and most preferably from about 0.4 to about 20.0 mg/kg body weight. Other dosages are viable; factors influencing dosage include, but are not limited to, the severity of the disease; previous treatment approaches; overall health of the patient; other diseases present, etc. The skilled artisan is readily credited with assessing a particular patient and determining a suitable dosage that falls within the ranges, or if necessary, outside of the ranges.

15

Introduction of the immunologically active chimeric anti-CD20 antibodies in these dose ranges can be carried out as a single treatment or over a series of treatments. With respect to chimeric antibodies, it is preferred that such introduction be carried out over a series of treatments; this preferred approach is predicated upon the treatment methodology associated with this disease. While not wishing to be bound by any particular theory, because the immunologically active chimeric anti-CD20 antibodies are both immunologically active and bind to CD20, upon initial introduction of the immunologically active chimeric anti-CD20 antibodies to the individual, peripheral blood B cell depletion will begin; we have observed a nearly complete depletion within about 24 hours post treatment infusion. Because of this, subsequent introduction(s) of the immunologically active chimeric anti-CD20 antibodies (or radiolabeled anti-CD20 antibodies) to the patient is presumed to: a) clear remaining peripheral blood B cells; b) begin B cell depletion from lymph nodes; c) begin B cell depletion

from other tissue sources, eg, bone marrow, tumor, etc. Stated again, by using repeated introductions of the immunologically active chimeric anti-CD20 antibodies, a series of events take place, each event being viewed by us as important to effective treatment of the disease. The first "event" then, can be viewed as principally directed to substantially depleting the patient's peripheral blood B cells; the subsequent "events" can be viewed as either principally directed to simultaneously or serially clearing remaining B cells from the system clearing lymph node B cells, or clearing other tissue B cells.

- 10 In effect, while a single dosage provides benefits and can be effectively utilized for disease treatment/management, a preferred treatment course can occur over several stages; most preferably, between about 0.4 and about 20 mg/kg body weight of the immunologically active chimeric anti-CD20 antibodies is introduced to the patient once a week for between about 2 to 10 weeks, most preferably for about 4 weeks.

With reference to the use of radiolabeled anti-CD20 antibodies, a preference is that the antibody is non-chimeric; this preference is predicted upon the significantly longer circulating half-life of chimeric antibodies vis-a-vis murine antibodies (*ie* with a longer circulating half-life, the radionuclide is present in the patient for extended periods). However, radiolabeled chimeric antibodies can be beneficially utilized with lower milli-Curies ("mCi") dosages used in conjunction with the chimeric antibody relative to the murine antibody. This scenario allows for a decrease in bone marrow toxicity to an acceptable level, while maintaining therapeutic utility.

A variety of radionuclides are applicable to the present invention and those skilled in the art are credited with the ability to readily determine which radionuclide is most appropriate under a variety of circumstances. For example,

iodine [131] is a well known radionuclide used for targeted immunotherapy. However, the clinical usefulness of iodine [131] can be limited by several factors including: eight-day physical half-life; dehalogenation of iodinated antibody both in the blood and at tumor sites; and emission characteristics (*eg* large gamma component) which can be suboptimal for localized dose deposition in tumor. With the advent of superior chelating agents, the opportunity for attaching metal chelating groups to proteins has increased the opportunities to utilize other radionuclides such as indium [131] and yttrium [90]. Yttrium [90] provides several benefits for utilization in radioimmunotherapeutic applications: the 64 hour half-life of yttrium [90] is long enough to allow antibody accumulation by tumor and, unlike *eg* iodine [131], yttrium [90] is a pure beta emitter of high energy with no accompanying gamma irradiation in its decay, with a range in tissue of 100 to 1000 cell diameters. Furthermore, the minimal amount of penetrating radiation allows for outpatient administration of yttrium [90]-labeled antibodies. Furthermore, internalization of labeled antibody is not required for cell killing, and the local emission of ionizing radiation should be lethal for adjacent tumor cells lacking the target antigen.

One non-therapeutic limitation to yttrium [90] is based upon the absence of significant gamma radiation making imaging therewith difficult. To avoid this problem, a diagnostic "imaging" radionuclide, such as indium [111], can be utilized for determining the location and relative size of a tumor prior to the administration of therapeutic doses of yttrium [90]-labeled anti-CD20. Indium [111] is particularly preferred as the diagnostic radionuclide because: between about 1 to about 10mCi can be safely administered without detectable toxicity; and the imaging data is generally predictive of subsequent yttrium [90]-labeled antibody distribution. Most imaging studies utilize 5mCi indium [111]-labeled antibody because this dose is both safe and has increased imaging efficiency compared with lower doses, with optimal imaging occurring at three to six days

after antibody administration. See, for example, Murray J.L. , 26 *J. Nuc. Med.* 3328 (1985) and Carragullo, J.A. et al , 26 *J. Nuc. Med.* 67 (1985).

Effective single treatment dosages (ie therapeutically effective amounts) of  
5 yttrium [90] labeled anti-CD20 antibodies range from between about 5 and about  
75mCi, more preferably between about 10 and about 40mCi. Effective single  
treatment non-marrow ablative dosages of iodine [131] labeled anti-CD20  
antibodies range from between about 5 and about 70mCi, more preferably  
between about 5 and about 40mCi. Effective single treatment ablative dosages  
10 (ie may require autologous bone marrow transplantation) of iodine [131] labeled  
anti-CD20 antibodies range from between about 30 and about 600mCi, more  
preferably between about 50 and less than about 500mCi. In conjunction with a  
chimeric anti-CD20 antibody, owing to the longer circulating half life vis-a-vis  
murine antibodies, an effective single treatment non-marrow ablative dosages of  
15 iodine [131] labeled chimeric anti-CD20 antibodies range from between about 5  
and about 40mCi, more preferably less than about 30mCi. Imaging criteria for,  
eg the indium [111] label, are typically less than about 5mCi.

With respect to radiolabeled anti-CD20 antibodies, therapy therewith can also  
20 occur using a single therapy treatment or using multiple treatments. Because of  
the radionuclide component, it is preferred that prior to treatment, peripheral  
stem cells ("PSC") or bone marrow ("BM") be "harvested" for patients  
experiencing potentially fatal bone marrow toxicity resulting from radiation. BM  
and/or PSC are harvested using standard techniques, and then purged and  
25 frozen for possible reinfusion. Additionally, it is most preferred that prior to  
treatment a diagnostic dosimetry study using a diagnostic labeled antibody (eg  
using indium [111]) be conducted on the patient, a purpose of which is to ensure  
that the therapeutically labeled antibody (eg using yttrium [90]) will not become  
unnecessarily "concentrated" in any normal organ or tissue.

Chimeric mouse/human antibodies have been described. See, for example, Morrison, S.L. *et al.*, *PNAS* 11:6851-6854 (November 1984); European Patent Publication No. 173494; Boulianne, G.L. *et al.*, *Nature* 312:643 (December 1984);  
5 Neubeiger, M.S. *et al.*, *Nature* 314:268 (March 1985); European Patent Publication No. 125023; Tan *et al.*, *J. Immunol.* 135:8564 (November 1985); Sun, L.K. *et al.*, *Hybridoma* 5/1:517 (1986); Sahagan *et al.*, *J. Immunol.* 137:1066-1074 (1986). See generally, Muron, *Nature* 312:597 (December 1984); Dickson, *Genetic Engineering News* 5/3 (March 1985); Marx, *Science* 229 455 (August  
10 1985); and Morrison *Science* 229:1202-1207 (September 1985). Robinson *et al.*, in PCT Publication Number WO 88/04936 describe a chimeric antibody with human constant region and murine variable region, having specificity to an epitope of CD20; the murine portion of the chimeric antibody of the Robinson references is derived from the 2H7 mouse monoclonal antibody (gamma 2b, kappa). While the  
15 reference notes that the described chimeric antibody is a "prime candidate" for the treatment of B cell disorders, this statement can be viewed as no more than a suggestion to those in the art to determine whether or not this suggestion is accurate for this particular antibody, particularly because the reference lacks any data to support an assertion of therapeutic effectiveness, and importantly,  
20 data using higher order mammals such as primates or humans.

Methodologies for generating chimeric antibodies are available to those in the art. For example, the light and heavy chains can be expressed separately, using, for example, immunoglobulin light chain and immunoglobulin heavy chains in  
25 separate plasmids. These can then be purified and assembled *in vitro* into complete antibodies; methodologies for accomplishing such assembly have been described. See, for example, Scharff, M., *Harvey Lectures* 69:125 (1974). *In vitro* reaction parameters for the formation of IgG antibodies from reduced isolated light and heavy chains have also been described. See, for example, Beychok, S.,

Cells of Immunoglobulin Synthesis, Academic Press, New York, p. 69, 1979. Co-expression of light and heavy chains in the same cells to achieve intracellular association and linkage of heavy and light chains into complete H<sub>2</sub>L<sub>2</sub> IgG antibodies is also possible. Such co-expression can be accomplished using either  
5 the same or different plasmids in the same host cell.

Another approach, and one which is our most preferred approach for developing a chimeric non-human/human anti-CD20 antibody, is based upon utilization of an expression vector which includes, *ab initio*, DNA encoding heavy and light  
10 chain constant regions from a human source. Such a vector allows for inserting DNA encoding non-human variable region such that a variety of non-human anti-CD20 antibodies can be generated, screened and analyzed for various characteristics (eg type of binding specificity, epitope binding regions, etc.); thereafter, cDNA encoding the light and heavy chain variable regions from a  
15 preferred or desired anti-CD20 antibody can be incorporated into the vector. We refer to these types of vectors as Tandem Chimeric Antibody Expression ("TCAE") vectors. A most preferred TCAE vector which was used to generate immunologically active chimeric anti-CD20 antibodies for therapeutic treatment of lymphomas is TCAE 8. TCAE 8 is a derivative of a vector owned by the  
20 assignee of this patent document, referred to as TCAE 5.2 the difference being that in TCAE 5.2, the translation initiation start site of the dominant selectable marker (neomycin phosphotransferase, "NEO") is a consensus Kozak sequence, while for TCAE 8, this region is a partially impaired consensus Kozak sequence. Details regarding the impact of the initiation start site of the dominant  
25 selectable marker of the TCAE vectors (also referred to as "ANEX vector") vis-a-vis protein expression are disclosed in detail in the co-pending application filed herewith.

TCAE 8 comprises four (4) transcriptional cassettes, and these are in tandem order, *ie* a human immunoglobulin light chain absent a variable region; a human immunoglobulin heavy chain absent a variable region; DHFR; and NEO. Each transcriptional cassette contains its own eukaryotic promoter and  
5 polyadenylation region (reference is made to Figure 1 which is a diagrammatic representation of the TCAE 8 vector). Specifically:

1) the CMV promoter/enhancer in front of the immunoglobulin heavy chain is a truncated version of the promoter/enhancer in front of the light chain, from the  
10 Nhe I site at -350 to the Sst I site at -16 (*see*, 41 *Cell* 521, 1985).

2) a human immunoglobulin light chain constant region was derived via amplification of cDNA by a PCR reaction. In TCAE 8, this was the human immunoglobulin light chain kappa constant region (Kabat numbering, amino  
15 acids 108-214, allotype Km 3, (*see*, Kabat, E.A. "Sequences of proteins of immunological interest," NIH Publication, Fifth Ed. No. 91-3242, 1991)), and the human immunoglobulin heavy chain gamma 1 constant region (Kabat numbering amino acids 114-478, allotype Gmla, Gmlz). The light chain was isolated from normal human blood (IDEC Pharmaceuticals Corporation, La Jolla,  
20 CA); RNA therefrom was used to synthesize cDNA which was then amplified using PCR techniques (primers were derived vis-a-vis the consensus from Kabat). The heavy chain was isolated (using PCR techniques) from cDNA prepared from RNA which was in turn derived from cells transfected with a human IgG1 vector (*see*, 3 *Prot. Eng.* 531, 1990; vector pN $\gamma$ 162). Two amino acids  
25 were changed in the isolated human IgG1 to match the consensus amino acid sequence from Kabat, to wit: amino acid 225 was changed from valine to alanine (GTT to GCA), and amino acid 287 was changed from methionine to lysine (ATG to AAG);





As will be appreciated by those in the art, the TCAE vectors beneficially allow for substantially reducing the time in generating the immunologically active chimeric anti-CD20 antibodies. Generation and isolation of non-human light and heavy chain variable regions, followed by incorporation thereof within the human light chain constant transcriptional cassette and human heavy chain constant transcriptional cassette, allows for production of immunologically active chimeric anti-CD20 antibodies.

We have derived a most preferred non-human variable region with specificity to the CD20 antigen using a murine source and hybridoma technology. Using polymerase chain reaction ("PCR") techniques, the murine light and heavy variable regions were cloned directly into the TCAE 8 vector--this is the most preferred route for incorporation of the non-human variable region into the TCAE vector. This preference is principally predicated upon the efficiency of the PCR reaction and the accuracy of insertion. However, other equivalent procedures for accomplishing this task are available. For example, using TCAE 8 (or an equivalent vector), the sequence of the variable region of a non-human anti-CD20 antibody can be obtained, followed by oligonucleotide synthesis of portions of the sequence or, if appropriate, the entire sequence; thereafter, the portions or the entire synthetic sequence can be inserted into the appropriate locations within the vector. Those skilled in the art are credited with the ability to accomplish this task.

Our most preferred immunologically active chimeric anti-CD20 antibodies were derived from utilization of TCAE 8 vector which included murine variable regions derived from monoclonal antibody to CD20; this antibody (to be discussed in detail, *infra*), is referred to as "2B8." The complete sequence of the variable regions obtained from 2B8 in TCAE 8 ("anti-CD20 in TCAE 8") is set forth in Figure 3 (SEQ. ID. NO. 2).

The host cell line utilized for protein expression is most preferably of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, DG44 and DUXB11 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CVI with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/O (mouse myeloma), P3x63-Ag3.653 (mouse myeloma), BFA-1clBPT (bovine endothelial cells), RAJI (human lymphocyte) and 293 (human kidney). Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

- 15 Preferably the host cell line is either DG44 ("CHO") or SP2/O. See Urland, G. *et al.*, "Effect of gamma rays and the dihydrofolate reductase locus: deletions and inversions." *Som. Cell & Mol. Gen.* 12/6:555-566 (1986), and Shulman, M. *et al.*, "A better cell line for making hybridomas secreting specific antibodies." *Nature* 276:269 (1978), respectively. Most preferably, the host cell line is DG44.
- 20 Transfection of the plasmid into the host cell can be accomplished by any technique available to those in the art. These include, but are not limited to, transfection (including electrophoresis and electroporation), cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A.A.G. "Mammalian Expression Vectors." Chapter 24.2, pp. 470-472 *Vectors*, Rodriguez and Denhardt, Eds. (Butterworths, Boston, MA 1988). Most
- 25 preferably, plasmid introduction into the host is via electroporation.

## F. EXAMPLES

The following examples are not intended, nor are they to be construed, as limiting the invention. The examples are intended to evidence: dose-imaging  
5 using a radiolabeled anti-CD20 antibody ("I2B8"); radiolabeled anti-CD20 antibody ("Y2B8"); and immunologically active, chimeric anti-CD20 antibody ("C2B8") derived utilizing a specific vector ("TCAE 8") and variable regions derived from murine anti-CD20 monoclonal antibody ("2B8").

### 10 I. RADIOLABELED ANTI-CD20 ANTIBODY 2B8

#### A. Anti-CD20 Monoclonal Antibody (Murine) Production ("2B8")

BALB/C mice were repeatedly immunized with the human lymphoblastoid cell line SB (*see*, Adams, R.A. *et al.*, "Direct implantation and serial  
15 transplantation of human acute lymphoblastic leukemia in hamsters, SB-2." *Can Res* 28:1121-1125 (1968); this cell line is available from the American Tissue Culture Collection, Rockville, MD., under ATCC accession number ATCC CCL 120), with weekly injections over a period of 3-4 months. Mice evidencing high serum titers of anti-CD20 antibodies, as determined by inhibition of known  
20 CD20-specific antibodies (anti-CD20 antibodies utilized were Leu 16, Beckton Dickinson, San Jose, CA, Cat. No. 7670; and B1, Coulter Corp., Hialeah, FL, Cat. No. 6602201) were identified; the spleens of such mice were then removed. Spleen cells were fused with the mouse myeloma SP2/0 in accordance with the protocol described in Einfeld, D.A. *et al.*, (1988) *EMBO* 7:711 (SP2/0 has ATCC  
25 accession no. ATCC CRL 8006).

Assays for CD20 specificity were accomplished by radioimmunoassay. Briefly, purified anti-CD20 B1 was radiolabeled with I<sup>125</sup> by the iodobead method as described in Valentine, M.A. *et al.*, (1989) *J. Biol. Chem.* 264:11282. (I<sup>125</sup>

Sodium Iodide, ICN, Irvine, CA, Cat. No. 28665H). Hybridomas were screened by co-incubation of 0.05 ml of media from each of the fusion wells together with 0.05 ml of I<sup>125</sup> labeled anti-CD20 B1 (10 ng) in 1% BSA, PBS (pH 7.4), and 0.5 ml of the same buffer containing 100,000 SB cells. After incubation for 1 hr at room temperature, the cells were harvested by transferring to 96 well titer plates (V&P Scientific, San Diego, CA), and washed thoroughly. Duplicate wells containing unlabeled anti-CD20 B1 and wells containing no inhibiting antibody were used as positive and negative controls, respectively. Wells containing greater than 50% inhibition were expanded and cloned. The antibody demonstrating the highest inhibition was derived from the cloned cell line designated herein as "2B8."

B. Preparation of 2B8-MX-DTPA Conjugate

i. MX-DTPA

Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid ("carbon-14 labeled MX-DTPA") was used as a chelating agent for conjugation of radiolabel to 2B8. Manipulations of MX-DTPA were conducted to maintain metal-free conditions, *ie* metal-free reagents were utilized and, when possible, polypropylene plastic containers (flasks, beakers, graduated cylinders, pipette tips) washed with Alconox and rinsed with Milli-Q water, were similarly utilized. MX-DTPA was obtained as a dry solid from Dr. Otto Gansow (National Institute of Health, Bethesda, MD) and stored desiccated at 4°C (protected from light), with stock solutions being prepared in Milli-Q water at a concentration of 2-5mM, with storage at -70°C. MX-DTPA was also obtained from Coulter Immunology (Hialeah, Florida) as the disodium salt in water and stored at -70°C.

ii. Preparation of 2B8

Purified 2B8 was prepared for conjugation with MX-DTPA by transferring the antibody into metal-free 50mM bicine-NaOff, pH 8.6, containing 150 mM NaCl, using repetitive buffer exchange with CENTRICON 30™ spin  
5 filters (30,000D, MWCO; Amicon). Generally, 50-200 µL of protein (10 mg/nl) was added to the filter unit, followed by 2 mL of bicine buffer. The filter was centrifuged at 4°C in a Sorval SS-34 rotor (6,000 rpm, 45 min.). Retentate volume was approximately 50-100 µL; this process was repeated twice using the same filter. Retentate was transferred to a polypropylene 1.5 mL screw cap tube,  
10 assayed for protein, diluted to 10.0 mg/mL and stored at 4°C until utilized; protein was similarly transferred into 50 mM sodium citrate, pH 5.5, containing 150 mM NaCl and 0.05% sodium azide, using the foregoing protocol.

iii. Conjugation of 2B8 with MX-DTPA

15 Conjugation of 2B8 with MX-DTPA was performed in polypropylene tubes at ambient temperature. Frozen MX-DTPA stock solutions were thawed immediately prior to use. 50-200 mL of protein at 10 mg/mL were reacted with MX-DTPA at a molar ratio of MX-DTPA-to-2B8 of 4:1. Reactions were initiated by adding the MX-DTPA stock solution and gently mixing; the conjugation was  
20 allowed to proceed overnight (14 to 20 hr), at ambient temperature. Unreacted MX-DTPA was removed from the conjugate by dialysis or repetitive ultrafiltration, as described above in Example I.B.ii, into metal-free normal saline (0.9% w/v) containing 0.05% sodium azide. The protein concentration was adjusted to 10 mg/mL and stored at 4°C in a polypropylene tube until  
25 radiolabeled.

iv. Determination of MX-DTPA Incorporation

MX-DTPA incorporation was determined by scintillation counting and comparing the value obtained with the purified conjugate to the specific

activity of the carbon-[14]-labeled MX-DTPA. For certain studies, in which non-radioactive MX-DTPA (Coulter Immunology) was utilized, MX-DTPA incorporation was assessed by incubating the conjugate with an excess of a radioactive carrier solution of yttrium-[90] of known concentration and specific activity.

A stock solution of yttrium chloride of known concentration was prepared in metal-free 0.05 N HCl to which carrier-free yttrium-[90] (chloride salt) was added. An aliquot of this solution was analyzed by liquid scintillation counting to determine an accurate specific activity for this reagent. A volume of the yttrium chloride reagent equal to 3-times the number of mols of chelate expected to be attached to the antibody, (typically 2 mol/mol antibody), was added to a polypropylene tube, and the pH adjusted to 4.0-4.5 with 2 M sodium acetate. Conjugated antibody was subsequently added and the mixture incubated 15-30 min. at ambient temperature. The reaction was quenched by adding 20 mM EDTA to a final concentration of 1 mM and the pH of the solution adjusted to approximately pH 6 with 2M sodium acetate.

After a 5 min. incubation, the entire volume was purified by high-performance, size-exclusion chromatography (described *infra*). The eluted protein-containing fractions were combined, the protein concentration determined, and an aliquot assayed for radioactivity. The chelate incorporation was calculated using the specific activity of the yttrium-[90] chloride preparation and the protein concentration.

#### v. Immunoreactivity of 2B8-MX-DTPA

The immunoreactivity of conjugated 2B8 was assessed using whole-cell ELISA. Mid-log phase SB cells were harvested from culture by centrifugation and washed two times with 1X HBSS. Cells were diluted to 1-2 X

10<sup>6</sup> cells/mL in HBSS and aliquoted into 96-well polystyrene microtiter plates at 50,000-100,000 cells/well. The plates were dried under vacuum for 2 h. at 40-45°C to fix the cells to the plastic; plates were stored dry at -20°C until utilized. For assay, the plates were warmed to ambient temperature immediately before use, then blocked with 1X PBS, pH 7.2-7.4 containing 1% BSA (2 h). Samples for assay were diluted in 1X PBS/1% BSA, applied to plates and serially diluted (1:2) into the same buffer. After incubating plates for 1 h. at ambient temperature, the plates were washed three times with 1X PBS. Secondary antibody (goat anti-mouse IgG1-specific HRP conjugate 50 µL) was added to wells (1:1500 dilution in 1X PBS/1% BSA) and incubated 1 h. at ambient temperature. Plates were washed four times with 1X PBS followed by the addition of ABTS substrate solution (50 mM sodium citrate, pH 4.5 containing 0.01% ATBS and 0.001% H<sub>2</sub>O<sub>2</sub>). Plates were read at 405 nm after 15-30 min. incubation. Antigen-negative HSB cells were included in assays to monitor non-specific binding. Immunoreactivity of the conjugate was calculated by plotting the absorbance values vs. the respective dilution factor and comparing these to values obtained using native antibody (representing 100% immunoreactivity) tested on the same plate; several values on the linear portion of the titration profile were compared and a mean value determined (data not shown).

20

vi. Preparation of Indium-[111]-Labeled 2B8-MX-DTPA ("I2B8")

Conjugates were radiolabeled with carrier-free indium-[111]. An aliquot of isotope (0.1-2 mCi/mg antibody) in 0.05 M HCL was transferred to a polypropylene tube and approximately one-tenth volume of metal-free 2 M HCL added. After incubation for 5 min., metal-free 2 M sodium acetate was added to adjust the solution to pH 4.0-4.4. Approximately 0.5 mg of 2B8-MX-DTPA was added from a stock solution of 10.0 mg/mL DTPA in normal saline, or 50 mM sodium citrate/150 mM NaCl containing 0.05% sodium azide, and the solution gently mixed immediately. The pH solution was checked with pH paper to verify



a value of 4.0-4.5 and the mixture incubated at ambient temperature for 15-30 min. Subsequently, the reaction was quenched by adding 20 mM EDTA to a final concentration of 1 mM and the reaction mixture was adjusted to approximately pH 6.0 using 2 M sodium acetate.

5

After a 5-10 min. incubation, uncomplexed radioisotope was removed by size-exclusion chromatography. The HPLC unit consisted of Waters Model 6000 or TosoHaas Model TSK-6110 solvent delivery system fitted, respectively, with a Waters U6K or Rheodyne 700 injection valve. Chromatographic separations  
10 were performed using a gel permeation column (BioRad SEC-250; 7.5 x 300 mm or comparable TosoHaas column) and a SEC-250 guard column (7.5 x 100 mm). The system was equipped with a fraction collector (Pharmacia Frac200) and a UV monitor fitted with a 280 nm filter (Pharmacia model UV-1). Samples were applied and eluted isocratically using 1X PBS, pH 7.4, at 1.0 mL/min flow rate.  
15 One-half milliliter fractions were collected in glass tubes and aliquots of these counted in a gamma counter. The lower and upper windows were set to 100 and 500 KeV respectively.

The radioincorporation was calculated by summing the radioactivity associated  
20 with the eluted protein peak and dividing this number by the total radioactivity eluted from the column; this value was then expressed as a percentage (data not shown). In some cases, the radioincorporation was determined using instant thin-layer chromatography ("ITLC"). Radiolabeled conjugate was diluted 1:10 or 1:20 in 1X PBS containing or 1X PBS/1 mM DTPA, then 1  $\mu$ L was spotted 1.5 cm  
25 from one end of a 1 x 5 cm strip of ITLC SG paper. The paper was developed by ascending chromatography using 10% ammonium acetate in methanol:water (1:1;v/v). The strip was dried, cut in half crosswise, and the radioactivity associated with each section determined by gamma counting. The radioactivity associated with the bottom half of the strip (protein-associated radioactivity) was

expressed as a percentage of the total radioactivity, determined by summing the values for both top and bottom halves (data not shown).

Specific activities were determined by measuring the radioactivity of an appropriate aliquot of the radiolabeled conjugate. This value was corrected for the counter efficiency (typically 75%) and related to the protein concentration of the conjugate, previously determined by absorbance at 280 nm, and the resulting value expressed as mCi/mg protein.

For some experiments, 2B8-MX-DTPA was radiolabeled with indium [111] following a protocol similar to the one described above but without purification by HPLC; this was referred to as the "mix-and-shoot" protocol.

vii. Preparation of Yttrium-[90]-Labeled 2B8-MX-DTPA ("Y2B8")

The same protocol described for the preparation of I2B8 was followed for the preparation of the yttrium-[90]-labeled 2B8-MX-DTPA ("Y2B8") conjugate except that 2 ng HCl was not utilized; all preparations of yttrium-labeled conjugates were purified by size-exclusion chromatography as described above.

20

C. Non-Human Animal Studies.

i. Biodistribution of Radiolabeled 2B8-MX-DTPA

I2B8 was evaluated for tissue biodistribution in six-to-eight week old BALB/c mice. The radiolabeled conjugate was prepared using clinical-grade 2B8-MX-DTPA following the "mix and shoot" protocol described above. The specific activity of the conjugate was 2.3 mCi/mg and the conjugate was formulated in PBS, pH 7.4 containing 50mg/mL HSA. Mice were injected intravenously with 100  $\mu$ L of I2B8 (approximately 21  $\mu$ Ci) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After

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sacrifice, the tail, heart, lungs, liver, kidney, spleen, muscle, and femur were removed, washed and weighed; a sample of blood was also removed for analysis. Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue subsequently determined. No  
5 attempt was made to discount the activity contribution represented by the blood associated with individual organs.

In a separate protocol, aliquots of 2B8-MX-DTPA incubated at 4°C and 30°C for 10 weeks were radiolabeled with indium-[111] to a specific activity of 2.1 mCi/mg  
10 for both preparations. These conjugates were then used in biodistribution studies in mice as described above.

For dosimetry determinations, 2B8-MX-DTPA was radiolabeled with indium-[111] to a specific activity of 2.3 mCi/mg and approximately 1.1  $\mu$ Ci was injected  
15 into each of 20 BALB/c mice. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs removed and prepared for analysis. In addition, portions of the skin, muscle and bone were removed and processed for analysis; the urine and feces were also collected and analyzed for the 24-72 hour time points.

20

Using a similar approach, 2B8-MX-DTPA was also radiolabeled with yttrium-[90] and its biological distribution evaluated in BALB/c mice over a 72-hour time period. Following purification by HPLC size exclusion chromatography, four groups of five mice each were injected intravenously with approximately 1  $\mu$ Ci of  
25 clinically-formulated conjugate (specific activity: 12.2 mCi/mg); groups were subsequently sacrificed at 1, 24, 48 and 72 hours and their organs and tissues analyzed as described above. Radioactivity associated with each tissue specimen was determined by measuring bremsstrahlung energy with a gamma scintillation counter. Activity values were subsequently expressed as percent injected dose

per gram tissue or percent injected dose per organ. While organs and other tissues were rinsed repeatedly to remove superficial blood, the organs were not perfused. Thus, organ activity values were not discounted for the activity contribution represented by internally associated blood.

5

ii. Tumor Localization of I2B8

The localization of radiolabeled 2B8-MX-DTPA was determined in athymic mice bearing Ramos B cell tumors. Six-to-eight week old athymic mice were injected subcutaneously (left-rear flank) with 0.1 mL of RPMI-1640  
10 containing  $1.2 \times 10^7$  Ramos tumor cells which had been previously adapted for growth in athymic mice. Tumors arose within two weeks and ranged in weight from 0.07 to 1.1 grams. Mice were injected intravenously with 100  $\mu$ L of indium-[111]-labeled 2B8-MX-DTPA (16.7  $\mu$ Ci) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After sacrifice the tail, heart,  
15 lungs, liver, kidney, spleen, muscle, femur, and tumor were removed, washed, weighed; a sample of blood was also removed for analysis. Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue determined.

20

iii. Biodistribution and Tumor Localization Studies with Radiolabeled 2B8-MX-DTPA

Following the preliminary biodistribution experiment described above (Example I.B.viii.a.), conjugated 2B8 was radiolabeled with indium-[111] to a specific activity of 2.3 mCi/mg and roughly 1.1  $\mu$ Ci was injected into each of  
25 twenty BALB/c mice to determine biodistribution of the radiolabeled material. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs and a portion of the skin, muscle and bone were removed and processed for analysis. In addition, the urine and feces were collected and analyzed for the 24-72 hour time-points. The level of radioactivity in the blood  
30 dropped from 40.3% of the injected dose per gram at 1 hour to 18.9% at 72 hours

(data not shown). Values for the heart, kidney, muscle and spleen remained in the range of 0.7-9.8% throughout the experiment. Levels of radioactivity found in the lungs decreased from 14.2% at 1 hour to 7.6% at 72 hours; similarly the respective liver injected-dose per gram values were 10.3% and 9.9%. These data were used in determining radiation absorbed dose estimates I2B8 described below.

The biodistribution of yttrium-[90]-labeled conjugate, having a specific activity of 12.2 mCi/mg antibody, was evaluated in BALB/c mice. Radioincorporations of >90% were obtained and the radiolabeled antibody was purified by HPLC. Tissue deposition of radioactivity was evaluated in the major organs, and the skin, muscle, bone, and urine and feces over 72 hours and expressed as percent injected dose/g tissue. Results (not shown) evidenced that while the levels of radioactivity associated with the blood dropped from approximately 39.2% injected dose per gram at 1 hour to roughly 15.4% after 72 hours the levels of radioactivity associated with tail, heart, kidney, muscle and spleen remained fairly constant at 10.2% or less throughout the course of the experiment. Importantly, the radioactivity associated with the bone ranged from 4.4% of the injected dose per gram bone at 1 hour to 3.2% at 72 hours. Taken together, these results suggest that little free yttrium was associated with the conjugate and that little free radiometal was released during the course of the study. These data were used in determining radiation absorbed dose estimates for Y2B8 described below.

For tumor localization studies, 2B8-MX-DTPA was prepared and radiolabeled with  $^{111}\text{In}$  to a specific activity of 2.7 mCi/mg. One hundred microliters of labeled conjugate (approximately 24  $\mu\text{Ci}$ ) were subsequently injected into each of 12 athymic mice bearing Ramos B cell tumors. Tumors ranged in weight from 0.1 to 1.0 grams. At time points of 0, 24, 48, and 72 hours following injection, 50

μL of blood was removed by retro-orbital puncture, the mice sacrificed by cervical dislocation, and the tail, heart, lungs, liver, kidney, spleen, muscle, femur, and tumor removed. After processing and weighing the tissues, the radioactivity associated with each tissue specimen was determined using a gamma counter and the values expressed as percent injected dose per gram.

The results (not shown) evidenced that the tumor concentrations of the <sup>111</sup>In-2B8-MX-DTPA increased steadily throughout the course of the experiment. Thirteen percent of the injected dose was accumulated in the tumor after 72 hours. The blood levels, by contrast, dropped during the experiment from over 30% at time zero to 13% at 72 hours. All other tissues (except muscle) contained between 1.3 and 6.0% of the injected dose per gram tissue by the end of the experiment; muscle tissue contained approximately 13% of the injected dose per gram.

#### D. Human Studies

##### i. 2B8 and 2B8-MX-DTPA: Immunohistology Studies with Human Tissues

The tissue reactivity of murine monoclonal antibody 2B8 was evaluated using a panel of 32 different human tissues fixed with acetone. Antibody 2B8 reacts with the anti-CD20 antigen which had a very restricted pattern of tissue distribution, being observed only in a subset of cells in lymphoid tissues including those of hematopoietic origin.

In the lymph node, immunoreactivity was observed in a population of mature cortical B-lymphocytes as well as proliferating cells in the germinal centers. Positive reactivity was also observed in the peripheral blood, B-cell areas of the tonsils, white pulp of the spleen, and with 40-70% of the medullary lymphocytes found in the thymus. Positive reactivity was also seen in the follicles of the

lamina propria (Peyer's Patches) of the large intestines. Finally, aggregates or scattered lymphoid cells in the stroma of various organs, including the bladder, breast, cervix, esophagus, lung, parotid, prostate, small intestine, and stomach, were also positive with antibody 2B8 (data not shown).

5

All simple epithelial cells, as well as the stratified epithelia and epithelia of different organs, were found to be unreactive. Similarly, no reactivity was seen with neuroectodermal cells, including those in the brain, spinal cord and peripheral nerves. Mesenchymal elements, such as skeletal and smooth muscle cells, fibroblasts, endothelial cells, and polymorphonuclear inflammatory cells were also found to be negative (data not shown).

The tissue reactivity of the 2B8-MX-DTPA conjugate was evaluated using a panel of sixteen human tissues which had been fixed with acetone. As previously demonstrated with the native antibody (data not shown), the 2B8-MX-DTPA conjugate recognized the CD20 antigen which exhibited a highly restricted pattern of distribution, being found only on a subset of cells of lymphoid origin. In the lymph node, immunoreactivity was observed in the B cell population. Strong reactivity was seen in the white pulp of the spleen and in the medullary lymphocytes of the thymus. Immunoreactivity was also observed in scattered lymphocytes in the bladder, heart, large intestines, liver, lung, and uterus, and was attributed to the presence of inflammatory cells present in these tissues. As with the native antibody, no reactivity was observed with neuroectodermal cells or with mesenchymal elements (data not shown).

25

ii. Clinical Analysis of I2B8 (Imaging) and Y2B8 (Therapy)

a. Phase I/II Clinical Trial Single Dose Therapy Study

A Phase I/II clinical analysis of I2B8 (imaging) followed by treatment with a single therapeutic dose of Y2B8 is currently being conducted.

5 For the single-dose study, the following schema is being followed:

1. Peripheral Stem Cell (PSC) or Bone Marrow (BM) Harvest with Purging;
2. I2B8 Imaging;
3. Y2B8 Therapy (three Dose Levels); and
- 10 4. PSC or Autologous BM Transplantation (if necessary based upon absolute neutrophil count below  $500/\text{mm}^3$  for three consecutive days or platelets below  $20,000/\text{mm}^3$  with no evidence of marrow recovery on bone marrow examination).

15 The Dose Levels of Y2B8 are as follows:

<u>Dose Level</u>	<u>Dose (mCi)</u>
1.	20
2.	30
3.	40

20 Three patients are to be treated at each of the dose levels for determination of a Maximum Tolerated Dose ("MTD").

Imaging (Dosimetry) Studies are conducted as follows: each patient is involved in two *in vivo* biodistribution studies using I2B8. In the first study, 2mg of I2B8 (5mCi), is administered as an intravenous (i.v.) infusion over one hour; one week later 2B8 (*ie* unconjugated antibody) is administered by i.v. at a rate not to exceed 250mg/hr followed immediately by 2mg of I2B8 (5mCi) administered by i.v. over one hour. In both studies, immediately following the I2B8 infusion, each patient is imaged and imaging is repeated at time  $t = 14-18$  hr (if indicated),  $t =$   
25 24 hr;  $t = 72$  hr; and  $t = 96$  hr (if indicated). Whole body average retention times for the indium [111] label are determined; such determinations are also made for recognizable organs or tumor lesions ("regions of interest").  
30



The regions of interest are compared to the whole body concentrations of the label; based upon this comparison, an estimate of the localization and concentration of Y2B8 can be determined using standard protocols. If the  
5 estimated cumulative dose of Y2B8 is greater than eight (8) times the estimated whole body dose, or if the estimated cumulative dose for the liver exceeds 1500 cGy, no treatment with Y2B8 should occur.

If the imaging studies are acceptable, either 0.0 or 1.0mg/kg patient body weight  
10 of 2B8 is administered by i.v. infusion at a rate not to exceed 250mg/h. This is followed by administration of Y2B8 (10,20 or 40mCi) at an i.v. infusion rate of 20mCi/hr.

b. Phase I/II Clinical Trial: Multiple Dose Therapy Study

15 A Phase I/II clinical analysis of of Y2B8 is currently being conducted. For the multiple-dose study, the following schema is being followed:

1. PSC or BM Harvest;
2. I2B8 Imaging;
- 20 3. Y2B8 Therapy (three Dose Levels) for four doses or a total cumulative dose of 80mCi; and
4. PSC or Autologous BM Transplantation (based upon decision of medical practitioner).

25 The Dose Levels of Y2B8 are as follows:

<u>Dose Level</u>	<u>Dose (mCi)</u>
1.	10
2.	15
3.	20

30 Three patients are to be treated at each of the dose levels for determination of an MTD.

Imaging (Dosimetry) Studies are conducted as follows: A preferred imaging dose for the unlabeled antibody (*ie* 2B8) will be determined with the first two patients. The first two patients will receive 100mg of unlabeled 2B8 in 250cc of normal saline over 4 hrs followed by 0.5mCi of I2B8 -- blood will be sampled for

5 biodistribution data at times  $t = 0$ ,  $t = 10$  min.,  $t = 120$  min.,  $t = 24$  hr, and  $t = 48$  hr. Patients will be scanned with multiple regional gamma camera images at times  $t = 2$  hr,  $t = 24$  hr and  $t = 48$  hr. After scanning at  $t = 48$  hr, the patients will receive 250mg of 2B8 as described, followed by 4.5mCi of I2B8 -- blood and scanning will then follow as described. If 100mg of 2B8 produces superior

10 imaging, then the next two patients will receive 50mg of 2B8 as described, followed by 0.5mCi of I2B8 followed 48 hrs later by 100mg 2B8 and then with 4.5mCi of I2B8. If 250mg of 2B8 produces superior imaging, then the next two patients will receive 250mg of 2B8 as described, followed by 0.5mCi of I2B8 followed 48 hrs later with 500mg 2B8 and then with 4.5mCi of I2B8. Subsequent

15 patients will be treated with the lowest amount of 2B8 that provides optimal imaging. Optimal imaging will be defined by: (1) best effective imaging with the slowest disappearance of antibody; (2) best distribution minimizing compartmentalization in a single organ; and (3) best subjective resolution of the lesion (tumor/background comparison).

20

For the first four patients, the first therapeutic dose of Y2B8 will begin 14 days after the last dose of I2B8; for subsequent patients, the first therapeutic dose of Y2B8 will begin between two to seven days after the I2B8.

25

Prior to treatment with Y2B8, for the patients other than the first four, 2B8 will be administered as described, followed by i.v. infusion of Y2B8 over 5-10 min. Blood will be sampled for biodistribution at times  $t = 0$ ,  $t = 10$  min.,  $t = 120$  min.,  $t = 24$  hr and  $t = 48$  hr. Patients will receive repetitive doses of Y2B8 (the same dose administered as with the first dose) approximately every six to eight weeks

for a maximum of four doses, or total cumulative dose of 80mCi. It is most preferred that patients not receive a subsequent dose of Y2B8 until the patients' WBC is greater than/equal to 3,000 and AGC is greater than/equal to 100,000.

- 5 Following completion of the three-dose level study, an MTD will be defined. Additional patients will then be enrolled in the study and these will receive the MTD.

10 II. CHIMERIC ANTI-CD20 ANTIBODY PRODUCTION ("C2B8")

A. Construction of Chimeric Anti-CD20 Immunoglobulin DNA Expression Vector

- 15 RNA was isolated from the 2B8 mouse hybridoma cell (as described in Chomczynski, P. *et al.*, "Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." *Anal. Biochem.* 162:156-159 (1987)). and cDNA was prepared therefrom. The mouse immunoglobulin light chain variable region DNA was isolated from the cDNA by polymerase chain reaction
- 20 using a set of DNA primers with homology to mouse light chain signal sequences at the 5' end and mouse light chain J region at the 3' end. Primer sequences were as follows:

1. V<sub>L</sub> Sense (SEQ. ID. NO. 3)

25

5' ATC AC AGATCT CTC ACC ATG GAT TTT CAG GTG CAG

ATT ATC AGC TTC 3'

- 30 (The underlined portion is a Bgl II site; the above-lined portion is the start codon.)

2. V<sub>L</sub> Antisense (SEQ. ID. NO. 4)

5' TGC AGC ATC CGTACG TTT GAT TTC CAG CTT 3'

(The underlined portion is a Bsi WI site.)

5

See, Figures 1 and 2 for the corresponding Bgl II and Bsi WI sites in TCAE 8, and Figure 3 for the corresponding sites in anti-CD20 in TCAE 8.

These resulting DNA fragment was cloned directly into the TCAE 8 vector in front of the human kappa light chain constant domain and sequenced. The determined DNA sequence for the murine variable region light chain is set forth in Figure 4 (SEQ. ID. NO. 5); *see also* Figure 3, nucleotides 978 through 1362. Figure 4 further provides the amino acid sequence from this murine variable region, and the CDR and framework regions. The mouse light chain variable region from 2B8 is in the mouse kappa VI family. *See, Kabat, supra.*

The mouse heavy chain variable region was similarly isolated and cloned in front of the human IgG1 constant domains. Primers were as follows:

20 1. V<sub>H</sub> Sense (SEQ. ID. NO. 6)

5' GCG GCT CCC ACGCGT GTC CTG TCC CAG 3'

(The underlined portion is an Mlu I site.)

25

2. V<sub>H</sub> Antisense (SEQ. ID. NO. 7)

5' GG(G/C) TGT TGT GCTAGC TG(A/C) (A/G)GA GAC  
(G/A)GT GA 3'

(The underlined portion is an Nhe I site.)

See, Figures 1 and 2 for corresponding Mlu I and Nhe I sites in TCAE 8, and  
5 Figure 3 for corresponding sites in anti-CD20 in TCAE 8.

The sequence for this mouse heavy chain is set forth in Figure 5 (SEQ. ID. NO.  
8); see also Figure 3, nucleotide 2401 through 2820. Figure 5 also provides the  
amino acid sequence from this murine variable region, and the CDR and  
10 framework regions. The mouse heavy chain variable region from 2B8 is in the  
mouse VH 2B family. See, Kabat, *supra*.

B. Creation of Chimeric Anti-CD20 Producing CHO and SP2/0  
Transfectomas

15 Chinese hamster ovary ("CHO") cells DG44 were grown in SSFM II minus  
hypoxanthine and thymidine media (Gibco, Grand Island, NY, Form No. 91-  
0456PK); SP2/0 mouse myeloma cells were grown in Dulbecco's Modified Eagles  
Medium media ("DMEM") (Irvine Scientific, Santa Ana, Ca., Cat. No. 9024) with  
5% fetal bovine serum and 20 ml/L glutamine added. Four million cells were  
20 electroporated with either 25 µg CHO or 50 µg SP2/0 plasmid DNA that had  
been restricted with Not I using a BTX 600 electroporation system (BTX, San  
Diego, CA) in 0.4 ml disposable cuvettes. Conditions were either 210 volts for  
CHO or 180 volts for SP2/0, 400 microfaradays, 13 ohms. Each electroporation  
was plated into six 96 well dishes (about 7,000 cells/well). Dishes were fed with  
25 media containing G418 (GENETICIN, Gibco, Cat. No. 860-1811) at 400 µg/ml  
active compound for CHO (media further included 50 µM hypoxanthine and 8  
µM thymidine) or 800 µg/ml for SP2/0, two days following electroporation and  
thereafter 2 or 3 days until colonies arose. Supernatant from colonies was  
assayed for the presence of chimeric immunoglobulin via an ELISA specific for  
30 human antibody. Colonies producing the highest amount of immunoglobulin

were expanded and plated into 96 well plates containing media plus methotrexate (25 nM for SP2/0 and 5nM for CHO) and fed every two or three days. Supernatants were assayed as above and colonies producing the highest amount of immunoglobulin were examined. Chimeric anti-CD20 antibody was  
5 purified from supernatant using protein A affinity chromatography.

Purified chimeric anti-CD20 was analyzed by electrophoresis in polyacrylamide gels and estimated to be greater than about 95% pure. Affinity and specificity of the chimeric antibody was determined based upon 2B8. Chimeric anti-CD20  
10 antibody tested in direct and competitive binding assays, when compared to murine anti-CD20 monoclonal antibody 2B8, evidenced comparable affinity and specificity on a number of CD20 positive B cells lines (data not presented). The apparent affinity constant ("Kap") of the chimeric antibody was determined by direct binding of I<sup>125</sup> radiolabeled chimeric anti-CD20 and compared to  
15 radiolabeled 2B8 by Scatchard plot; estimated Kap for CHO produced chimeric anti-CD20 was  $5.2 \times 10^{-9}$  M and for SP2/0 produced antibody,  $7.4 \times 10^{-9}$  M. The estimated Kap for 2B8 was  $3.5 \times 10^{-9}$  M. Direct competition by radioimmunoassay was utilized to confirm both the specificity and retention of immunoreactivity of the chimeric antibody by comparing its ability to effectively  
20 compete with 2B8. Substantially equivalent amounts of chimeric anti-CD20 and 2B8 antibodies were required to produce 50% inhibition of binding to CD20 antigens on B cells (data not presented), *ie* there was a minimal loss of inhibiting activity of the anti-CD20 antibodies, presumably due to chimerization.

25 The results of Example II.B indicate, *inter alia*, that chimeric anti-CD20 antibodies were generated from CHO and SP2/0 transfectomas using the TCAE 8 vectors, and these chimeric antibodies had substantially the same specificity and binding capability as murine anti-CD20 monoclonal antibody 2B8.

C. Determination of Immunological Activity of Chimeric Anti-CD20 Antibodies

i. Human C1q Analysis

Chimeric anti-CD20 antibodies produced by both CHO and SP2/0 cell lines were evaluated for human C1q binding in a flow cytometry assay using fluorescein labeled C1q (C1q was obtained from Quidel, Mira Mesa, CA, Prod. No. A400 and FITC label from Sigma, St. Louis MO, Prod. No. F-7250; FITC. Labeling of C1q was accomplished in accordance with the protocol described in *Selected Methods In Cellular Immunology*, Michell & Shiigi, Ed. (W.H. Freeman & Co., San Francisco, CA, 1980, p. 292). Analytical results were derived using a Becton Dickinson FACScan™ flow cytometer (fluorescein measured over a range of 515-545 nm). Equivalent amounts of chimeric anti-CD20 antibody, human IgG1,K myeloma protein (Binding Site, San Diego, Ca, Prod. No. BP078), and 2B8 were incubated with an equivalent number of CD20-positive SB cells, followed by a wash step with FACS buffer (.2% BSA in PBS, pH 7.4, .02% sodium azide) to remove unattached antibody, followed by incubation with FITC labeled C1q. Following a 30-60 min. incubation, cells were again washed. The three conditions, including FITC-labeled C1q as a control, were analyzed on the FACScan™ following manufacturing instructions. Results are presented in Figure 6.

As the results of Figure 6 evidence, a significant increase in fluorescence was observed only for the chimeric anti-CD20 antibody condition; ie only SB cells with adherent chimeric anti-CD20 antibody were C1q positive, while the other conditions produced the same pattern as the control.

ii. Complement Dependent Cell Lyses

Chimeric anti-CD20 antibodies were analyzed for their ability to lyse lymphoma cell lines in the presence of human serum (complement source). CD20 positive SB cells were labeled with  $^{51}\text{Cr}$  by admixing 100 $\mu$  Ci of  $^{51}\text{Cr}$  with

1x10<sup>6</sup> SB cells for 1 hr at 37°C; labeled SB cells were then incubated in the presence of equivalent amounts of human complement and equivalent amounts (0-50 µg/ml) of either chimeric anti-CD20 antibodies or 2B8 for 4 hrs at 37°C (see, Brunner, K.T. *et al.*, "Quantitative assay of the lytic action of immune lymphoid cells on <sup>51</sup>Cr-labeled allogeneic target cells *in vitro*." *Immunology* 14:181-189 (1968). Results are presented in Figure 7.

The results of Figure 7 indicate, *inter alia*, that chimeric anti-CD20 antibodies produced significant lysis (49%) under these conditions.

10

iii. Antibody Dependent Cellular Cytotoxicity Effector Assay

For this study, CD20 positive cells (SB) and CD20 negative cells (T cell leukemia line HSB; see, Adams, Richard, "Formal Discussion," *Can. Res.* 27:2479-2482 (1967); ATCC deposit no. ATCC CCL 120.1) were utilized; both were labeled with <sup>51</sup>Cr. Analysis was conducted following the protocol described in Brunner, K.T. *et al.*, "Quantitative assay of the lytic action of immune lymphoid cells on <sup>51</sup>Cr-labeled allogeneic target cells *in vitro*; inhibition by isoantibody and drugs." *Immunology* 14:181-189 (1968); a substantial chimeric anti-CD20 antibody dependent cell mediated lysis of CD20 positive SB target cells (<sup>51</sup>Cr-labeled) at the end of a 4 hr, 37°C incubation, was observed and this effect was observed for both CHO and SP2/0 produced antibody (effector cells were human peripheral lymphocytes; ratio of effector cells:target was 100:1). Efficient lysis of target cells was obtained at 3.9 µg/ml. In contrast, under the same conditions, the murine anti-CD20 monoclonal antibody 2B8 had a statistically insignificant effect, and CD20 negative HSB cells were not lysed. Results are presented in Figure 8.

The results of Example II indicate, *inter alia*, that the chimeric anti-CD20 antibodies of Example I were immunologically active.



### III. DEPLETION OF B CELLS *IN VIVO* USING CHIMERIC ANTI-CD20

#### A. Non-Human Primate Study

- 5 Three separate non-human primate studies were conducted. For convenience, these are referred to herein as "Chimeric Anti-CD20: CHO & SP2/0;" "Chimeric Anti-CD20: CHO;" and "High Dosage Chimeric Anti-CD20." Conditions were as follows:
- 10 Chimeric Anti-CD20: CHO & SP2/0
- Six cynomolgus monkeys ranging in weight from 4.5 to 7 kilograms (White Sands Research Center, Alamogordo, NM) were divided into three groups of two monkeys each. Both animals of each group received the same dose of immunologically active chimeric anti-CD20 antibody. One animal in each group
- 15 received purified antibody produced by the CHO transfectoma; the other received antibody produced by the SP2/0 transfectoma. The three groups received antibody dosages corresponding to 0.1 mg/kg, 0.4 mg/kg, and 1.6 mg/kg each day for four (4) consecutive days. The chimeric immunologically active anti-CD20 antibody, which was admixed with sterile saline, was administered by
- 20 intravenous infusion; blood samples were drawn prior to each infusion. Additional blood samples were drawn beginning 24 hrs after the last injection (T=0) and thereafter on days 1, 3, 7, 14 and 28; blood samples were also taken thereafter at biweekly intervals until completion of the study at day 90.
- 25 Approximately 5 ml of whole blood from each animal was centrifuged at 2000 RPM for 5 min. Plasma was removed for assay of soluble chimeric anti-CD20 antibody levels. The pellet (containing peripheral blood leukocytes and red blood cells) was resuspended in fetal calf serum for fluorescent-labeled antibody

analysis (*see*, "Fluorescent Antibody Labeling of Lymphoid Cell Population," *infra.*).

#### Chimeric Anti-CD20: CHO

- 5 Six cynomolgus monkeys ranging in weight from 4 to 6 kilograms (White Sands) were divided into three groups of two monkeys each. All animals were injected with immunologically active chimeric anti-CD20 antibodies produced from the CHO transfectoma (in sterile saline). The three groups were separated as follows: subgroup 1 received daily intravenous injections of 0.01 mg/kg of the
- 10 antibody over a four (4) day period; subgroup 2 received daily intravenous injections of 0.4 mg/kg of the antibody over a four (4) day period; subgroup 3 received a single intravenous injection of 6.4 mg/kg of the antibody. For all three subgroups, a blood sample was obtained prior to initiation of treatment; additionally, blood samples were also drawn at T=0, 1, 3, 7, 14 and 28 days
- 15 following the last injection, as described above, and these samples were processed for fluorescent labeled antibody analysis (*see*, "Fluorescent Antibody Labeling," *infra.*). In addition to peripheral blood B cell quantitation, lymph node biopsies were taken at days 7, 14 and 28 following the last injection, and a single cell preparation stained for quantitation of lymphocyte populations by flow
- 20 cytometry.

#### High Dosage Chimeric Anti-CD20

- Two cynomolgus monkeys (White Sands) were infused with 16.8 mg/kg of the immunologically active chimeric anti-CD20 antibodies from the CHO
- 25 transfectomas (in sterile saline) weekly over a period of four consecutive weeks. At the conclusion of the treatment, both animals were anesthetized for removal of bone marrow; lymph node biopsies were also taken. Both sets of tissue were stained for the presence of B lymphocytes using Leu 16 by flow cytometry following the protocol described in Ling, N.R. *et al.*, "B-cell and plasma cell

antigens." *Leucocyte Typing III White Cell Differentiations Antigens*, A.J. McMichael, Ed. (Oxford University Press, Oxford UK, 1987), p. 302.

#### Fluorescent Antibody Labeling of Lymphoid Cell Population

- 5 After removal of plasma, leukocytes were washed twice with Hanks Balanced Salt Solution ("HBSS") and resuspended in a plasma equivalent volume of fetal bovine serum (heat inactivated at 56°C for 30 min.). A 0.1 ml volume of the cell preparation was distributed to each of six (6), 15 ml conical centrifuge tubes
- 10 Fluorescein labeled monoclonal antibodies with specificity for the human lymphocyte surface markers CD2 (AMAC, Westbrook, ME), CD20 (Becton Dickinson) and human IgM (Binding Site, San Diego, CA) were added to 3 of the tubes for identifying T and B lymphocyte populations. All reagents had previously tested positive to the corresponding monkey lymphocyte antigens. Chimeric anti-CD20 antibody bound to monkey B cell surface CD20 was
- 15 measured in the fourth tube using polyclonal goat anti-human IgG coupled with phycoerythrin (AMAC). This reagent was pre-adsorbed on a monkey Ig-sepharose column to prevent cross-reactivity to monkey Ig, thus allowing specific detection and quantitation of chimeric anti-CD20 antibody bound to cells. A fifth tube included both anti-IgM and anti-human IgG reagents for double stained B
- 20 cell population. A sixth sample was included with no reagents for determination of autofluorescence. Cells were incubated with fluorescent antibodies for 30 min., washed and fixed with 0.5 ml of fixation buffer (0.15 M NaCl, 1% paraformaldehyde, pH7.4) and analyzed on a Becton Dickinson FACScan™ instrument. Lymphocyte populations were initially identified by forward versus
- 25 right angle light scatter in a dot-plot bitmap with unlabeled leucocytes. The total lymphocyte population was then isolated by gating out all other events. Subsequent fluorescence measurements reflected only gated lymphocyte specific events.

## Depletion of Peripheral Blood B Lymphocytes

No observable difference could be ascertained between the efficacy of CHO and SP2/0 produced antibodies in depleting B cells *in vivo*, although a slight increase in B cell recovery beginning after day 7 for monkeys injected with chimeric anti-CD20 antibodies derived from CHO transfectomas at dosage levels 1.6 mg/kg and 6.4 mg/kg was observed and for the monkey injected with SP2/0 producing antibody at the 0.4 mg/kg dose level. Figures 9A, B and C provide the results derived from the chimeric anti-CD20:CHO & SP2/0 study, with Figure 9A directed to the 0.4 mg/kg dose level; Figure 9B directed to the 1.6 mg/kg dose level; and Figure 9C directed to the 6.4 mg/kg dose level.

As is evident from Figure 9, there was a dramatic decrease (>95%) in peripheral B cell levels after the therapeutic treatment across all tested dose ranges, and these levels were maintained up to seven (7) days post infusion; after this period, B cell recovery began, and, the time of recovery initiation was independent of dosage levels.

In the Chimeric Anti-CD20:CHO study, a 10-fold lower antibody dosage concentration (0.01 mg/kg) over a period of four daily injections (0.04 mg/kg total) was utilized. Figure 10 provides the results of this study. This dosage depleted the peripheral blood B cell population to approximately 50% of normal levels estimated with either the anti-surface IgM or the Leu 16 antibody. The results also indicate that saturation of the CD20 antigen on the B lymphocyte population was not achieved with immunologically active chimeric anti-CD20 antibody at this dose concentration over this period of time for non-human primates; B lymphocytes coated with the antibody were detected in the blood samples during the initial three days following therapeutic treatment. However, by day 7, antibody coated cells were undetectable.

Table I summarizes the results of single and multiple doses of immunologically active chimeric anti-CD20 antibody on the peripheral blood populations; single dose condition was 6.4 mg/kg; multiple dose condition was 0.4 mg/kg over four (4) consecutive days (these results were derived from the monkeys described above).

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TABLE I  
PERIPHERAL BLOOD POPULATION FROM C2B8 PRIMATE STUDY

	<u>Monkey</u>	<u>Dose</u>	<u>Day</u>	<u>CD2</u>	<u>Anti-Hu IgG</u>
5	A	0.4 mg/kg (4 doses)	Prebleed 0 7 21	81.5 86.5 85.5 93.3	- 0.2 0.0 -
10			28	85.5	-
	B	0.4 mg/kg (4 doses)	Prebleed 0 7 21	81.7 94.6 92.2 84.9	- 0.1 0.1 -
15			28	84.1	-
	C	6.4 mg/kg (1 dose)	Prebleed 7 21 28	77.7 85.7 86.7 76.7	0.0 0.1 - -
20					
	D	6.4 mg/kg (1 dose)	Prebleed 7 21 28	85.7 94.7 85.2 85.9	0.1 0.1 - -
25					
	<u>Monkey</u>	<u>Anti-Hu IgG+ Anti-Hu IgM*</u>	<u>Leu-16</u>	<u>% B Cell Depletion</u>	
30	A	- 0.3 0.1 -	9.4 0.0 1.2 2.1	0 97 99 78	
35		-	4.1	66	
	B	- 0.2 0.1 -	14.8 0.1 0.1 6.9	0 99 99 53	
40		-	8.7	41	
	C	0.2 0.1 - -	17.0 0.0 14.7 8.1	0 99 15 62	
45					
	D	0.1 0.2 - -	14.4 0.0 9.2 6.7	0 99 46 53	
50					

\*Double staining population which indicates extent of chimeric anti-CD20 coated B cells.

The data summarized in Table I indicates that depletion of B cells in peripheral blood under conditions of antibody excess occurred rapidly and effectively, regardless of single or multiple dosage levels. Additionally, depletion was  
5 observed for at least seven (7) days following the last injection, with partial B cell recovery observed by day 21.

Table II summarizes the effect of immunologically active, chimeric anti-CD20 antibodies on cell populations of lymph nodes using the treatment regimen of  
10 Table I (4 daily doses of 0.4 mg/kg; 1 dose of 6.4 mg/kg); comparative values for normal lymph nodes (control monkey, axillary and inguinal) and normal bone marrow (two monkeys) are also provided.

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TABLE II  
CELL POPULATIONS OF LYMPH NODES

	<u>Monkey</u>	<u>Dose</u>	<u>Day</u>	<u>CD2</u>	<u>Anti-Hu IgM</u>
5	A	0.4 mg/kg (4 doses)	7 14 28	66.9 76.9 61.6	- 19.6 19.7
10	B	0.4 mg/kg (4 doses)	7 14 28	59.4 83.2 84.1	- 9.9 15.7
15	C	6.4 mg/kg (1 dose)	7 14 28	75.5 74.1 66.9	- 17.9 23.1
20	D	6.4 mg/kg (1 dose)	7 14 28	83.8 74.1 84.1	- 17.9 12.8

TABLE II (continued)

	<u>Monkey</u>	<u>Anti-Hu IgG + Anti-Hu IgM</u>	<u>Leu-16</u>	<u>% B Lymphocyte Depletion</u>
25	A	7.4 0.8 -	40.1 22.6 26.0	1 44 36
30	B	29.9 0.7 -	52.2 14.5 14.6	0 64 64
35	C	22.3 1.1 -	35.2 23.9 21.4	13 41 47
40	D	12.5 0.2 -	19.7 8.7 12.9	51 78 68

TABLE II (continued)

	<u>CD2</u>	<u>Anti-Hu IgG+ Anti-Hu IgM</u>	<u>Anti-Hu IgM</u>	<u>Leu-16</u>	<u>% B Lymphocyte Depletion</u>
45	Normal Lymph Nodes				
	Control 1				
	Axillary	55.4	25.0	-	41.4 NA
	Inguinal	52.1	31.2	-	39.5 NA
	Normal Bone Marrow				
50	Control 2	65.3	19.0	-	11.4 NA
	Control 3	29.8	28.0	-	16.6 NA



The results of Table II evidence effective depletion of B lymphocytes for both treatment regimens. Table II further indicates that for the non-human primates, complete saturation of the B cells in the lymphatic tissue with immunologically active, chimeric anti-CD20 antibody was not achieved; additionally, antibody coated cells were observed seven (7) days after treatment, followed by a marked depletion of lymph node B cells, observed on day 14.

Based upon this data, the single High Dosage Chimeric Anti-CD20 study referenced above was conducted, principally with an eye toward pharmacology/toxicology determination. *Ie* this study was conducted to evaluate any toxicity associated with the administration of the chimeric antibody, as well as the efficacy of B cell depletion from peripheral blood lymph nodes and bone marrow. Additionally, because the data of Table II indicates that for that study, the majority of lymph node B cells were depleted between 7 and 14 days following treatment, a weekly dosing regimen might evidence more efficacious results. Table III summarizes the results of the High Dosage Chimeric Anti-CD20 study.

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TABLE III  
CELL POPULATIONS OF LYMPH NODES AND BONE MARROW

5	Lymphocyte Populations (%)					
	<u>Monkey</u>	<u>CD2</u>	<u>CD20<sup>a</sup></u>	<u>mIgM + anti-C2B8<sup>b</sup></u>	<u>C2B8<sup>c</sup></u>	<u>Day<sup>d</sup></u>
	<u>Inguinal Lymph Node</u>					
10	E	90.0	5.3	4.8	6.5	22
	F	91.0	6.3	5.6	6.3	22
15	G	89.9	5.0	3.7	5.8	36
	H	85.4	12.3	1.7	1.8	36
20	<u>Bone Marrow</u>					
	E	46.7	4.3	2.6	2.8	22
	F	41.8	3.0	2.1	2.2	22
25	G	35.3	0.8	1.4	1.4	36
	H	25.6	4.4	4.3	4.4	36
30	<sup>a</sup> Indicates population stained with Leu 16.					
	<sup>b</sup> Indicates double staining population, positive for surface IgM cells and chimeric antibody coated cells.					
35	<sup>c</sup> Indicates total population staining for chimeric antibody including double staining surface IgM positive cells and single staining (surface IgM negative) cells.					
40	<sup>d</sup> Days after injection of final 16.8 mg/kg dose.					
	Both animals evaluated at 22 days post treatment cessation contained less than 5% B cells, as compared to 40% in control lymph nodes ( <i>see</i> , Table II, <i>supra</i> ). Similarly, in the bone marrow of animals treated with chimeric anti-CD20 antibody, the levels of CD20 positive cells were less than 3% as compared to 11-15% in the normal animals ( <i>see</i> , Table II, <i>supra</i> ). In the animals evaluated at 36 days post treatment cessation, one of the animals (H) had approximately 12% B cells in the lymph node and 4.4% B cells in bone marrow, while the other (G) had					

approximately 5% B cells in the lymph node and 0.8% in the bone marrow--the data is indicative of significant B cell depletion.

The results of Example III.A indicate, *inter alia*, that low doses of immunologically active, chimeric anti-CD20 leads to long-term peripheral blood B cell depletion in primates. The data also indicates that significant depletion of B cell populations was achieved in peripheral lymph nodes and bone marrow when repetitive high doses of the antibody were administered. Continued follow-up on the test animals has indicated that even with such severe depletion of peripheral B lymphocytes during the first week of treatment, no adverse health effects have been observed. Furthermore, as recovery of B cell population was observed, a conclusion to be drawn is that the pluripotent stem cells of these primates were not adversely affected by the treatment.

B. Clinical Analysis of C2B8

i. Phase I/II Clinical Trial of C2B8: Single Dose Therapy Study

Fifteen patients having histologically documented relapsed B cell lymphoma have been treated with C2B8 in a Phase I/II Clinical Trial. Each patient received a single dose of C2B8 in a dose-escalating study; there were three patients per dose: 10mg/m<sup>2</sup>; 50mg/m<sup>2</sup>; 100mg/m<sup>2</sup>; 250mg/m<sup>2</sup> and 500mg/m<sup>2</sup>. Treatment was by i.v. infusion through an 0.22 micron in-line filter with C2B8 being diluted in a final volume of 250cc or a maximal concentration of 1mg/ml of normal saline. Initial rate was 50cc/hr for the first hour; if no toxicity was seen, dose rate was able to be escalated to a maximum of 200cc/hr.

Toxicity (as indicated by the clinician) ranged from "none", to "fever" to "moderate" (two patients) to "severe" (one patient); all patients completed the therapy treatment. Peripheral Blood Lymphocytes were analyzed to determine, *inter alia*, the impact of C2B8 on T-cells and B-cells. Consistently for all

patients, Peripheral Blood B Lymphocytes were depleted after infusion with C2B8 and such depletion was maintained for in excess of two weeks.

One patient (receiving 100mg/m<sup>2</sup> of C2B8) evidenced a Partial Response to the C2B8 treatment (reduction of greater than 50% in the sum of the products of the perpendicular diameters of all measurable indicator lesions lasting greater than four weeks, during which no new lesions may appear and no existing lesions may enlarge); at least one other patient (receiving 500mg/m<sup>2</sup>) evidenced a Minor Response to the C2B8 treatment (reduction of less than 50% but at least 25% in the sum of the products of the two longest perpendicular diameters of all measurable indicator lesions). For presentational efficiency, results of the PBLs are set forth in Figure 14; data for the patient evidencing a PR is set forth in Figure 14A; for the patient evidencing an MR, data is set forth in Figure 14B. In Figure 14, the following are applicable: ■ = Lymphocytes; ■ = CD3+ cells (T cells); ▲ = CD20+ cells; ● = CD19+ cells; ⊖ = Kappa; △ = lambda; and ◆ = C2B8. As evidenced, the B cell markers CD20 and CD19, Kappa and Lambda, were depleted for a period in excess of two weeks; while there was a slight, initial reduction in T-cell counts, these returned to an approximate base-line level in a relatively rapid time-frame.

ii. Phase III Clinical Trial of C2B8: Multiple Dose Therapy Study

Patients having histologically confirmed B cell lymphoma with measurable progressive disease are eligible for this study which is separated into two parts: in Phase I, consisting of a dose escalation to characterize dose limiting toxicities and determination of biologically active tolerated dose level, groups of three patients will receive weekly i.v. infusions of C2B8 for a total of four (4) separate infusions. Cumulative dose at each of the three levels will be as follows: 500mg/m<sup>2</sup> (125mg/m<sup>2</sup>/infusion); 1000mg/m<sup>2</sup> (250mg/m<sup>2</sup>/infusion);

1500mg/m<sup>2</sup> (375mg/m<sup>2</sup>/infusion. A biologically active tolerated dose is defined, and will be determined, as the lowest dose with both tolerable toxicity and adequate activity); in Phase II, additional patients will receive the biologically active tolerated dose with an emphasis on determining the activity of the four  
5 doses of C2B8.

#### IV. COMBINATION THERAPY: C2B8 AND Y2B8

A combination therapeutic approach using C2B8 and Y2B8 was  
10 investigated in a mouse xenographic model (nu/nu mice, female, approximately 10 weeks old) utilizing a B cell lymphoblastic tumor (Ramos tumor cells). For comparative purposes, additional mice were also treated with C2B8 and Y2B8.

Ramos tumor cells (ATCC, CRL 1596) were maintained in culture using RPMI-  
15 1640 supplemented with 10% fetal calf serum and glutamine at 37°C and 5% CO<sub>2</sub>. Tumors were initiated in nine female nude mice approximately 7-10 weeks old by subcutaneous injection of  $1.7 \times 10^6$  Ramos cells in a volume of 0.10ml (HBSS) using a 1cc syringe fitted with 25g needle. All animals were manipulated in a laminar flow hood and all cages, bedding, food and water were  
20 autoclaved. Tumor cells were passaged by excising tumors and passing these through a 40 mesh screen; cells were washed twice with 1X HBSS (50ml) by centrifugation (1300RPM), resuspended in 1X HBSS to  $10 \times 10^6$  cells/ml, and frozen at -70°C until used.

25 For the experimental conditions, cells from several frozen lots were thawed, pelleted by centrifugation (1300RPM) and washed twice with 1X HBSS. Cells were then resuspended to approximately  $2.0 \times 10^6$  cells/ml. Approximately 9 to 12 mice were injected with 0.10ml of the cell suspension (s.c.) using a 1cc syringe fitted with a 25g needle; injections were made on the animal's left side.

approximately mid-region. Tumors developed in approximately two weeks. Tumors were excised and processed as described above. Study mice were injected as described above with  $1.67 \times 10^6$  cells in 0.10ml HBSS.

- 5 Based on preliminary dosing experiments, it was determined that 200mg of C2B8 and 100 $\mu$ Ci of Y2B8 would be utilized for the study. Ninety female nu/nu mice (approximately 10 weeks old) were injected with the tumor cells.

Approximately ten days later, 24 mice were assigned to four study groups (six mice/group) while attempting to maintain a comparable tumor size distribution in each group (average tumor size, expressed as a product of length x width of the tumor, was approximately 80mm<sup>2</sup>). The following groups were treated as indicated via tail-vein injections using a 100 $\mu$ l Hamilton syringe fitted with a 25g needle:

- 15        A.    Normal Saline  
          B.    Y2B8 (100 $\mu$ Ci)  
          C.    C2B8 (200 $\mu$ g); and  
          D.    Y2B8 (100 $\mu$ Ci) + C2B8 (200 $\mu$ g)

- 20 Groups tested with C2B8 were given a second C2B8 injection (200 $\mu$ g/mouse) seven days after the initial injection. Tumor measurements were made every two or three days using a caliper.

Preparation of treatment materials were in accordance with the following  
25 protocols:

A    Preparation of Y2B8

- Yttrium-[90] chloride (6mCi) was transformed to a polypropylene tube and adjusted to pH 4.1-4.4 using metal free 2M sodium acetate. 2B8-MX-DTPA  
30 (0.3mg in normal saline; see above for preparation of 2B8-MX-DTPA) was added

and gently mixed by vortexing. After 15 min. incubation, the reaction was quenched by adding 0.05 x volume 20mM EDTA and 0.05X volume 2M sodium acetate. Radioactivity concentration was determined by diluting 5.0µl of the reaction mixture in 2.5ml 1 x PBS containing 75mg/ml HSA and 1mM DTPA ("formulation buffer"); counting was accomplished by adding 10.0µl to 20ml of Ecolume™ scintillation cocktail. The remainder of the reactive mixture was added to 3.0ml formulation buffer, sterile filtered and stored at 2-8°C until used. Specific activity (14mCi/mg at time of injection) was calculated using the radioactivity concentration and the calculated protein concentration based upon the amount of antibody added to the reaction mixture. Protein-associated radioactivity was determined using instant thin-layer chromatography. Radioincorporation was 95%. Y2B8 was diluted in formulation buffer immediately before use and sterile-filtered (final radioactivity concentration was 1.0mCi/ml).

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B. Preparation of C2B8

C2B8 was prepared as described above. C2B8 was provided as a sterile reagent in normal saline at 5.0mg/ml. Prior to injection, the C2B8 was diluted in normal saline to 2.0mg/ml and sterile filtered.

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C. Results

Following treatment, tumor size was expressed as a product of length and width, and measurements were taken on the days indicated in Figure 11 (Y2B8 vs. Saline); Figure 12 (C2B8 vs. Saline); and Figure 13 (Y2B8 + C2B8 vs. Saline). Standard error was also determined.

25

As indicated in Figure 13, the combination of Y2B8 and C2B8 exhibited tumoricidal effects comparable to the effects evidenced by either Y2B8 or C2B8.

## V. ALTERNATIVE THERAPY STRATEGIES

Alternative therapeutic strategies recognized in view of the foregoing examples are evident. One such strategy employs the use of a therapeutic dose of C2B8 followed within about one week with a combination of either 2B8 and radioabeled 2B8 (*eg* Y2B8); or 2B8, C2B8 and, *eg* Y2B8; or C2B8 and, *eg* Y2B8. An additional strategy is utilization of radiolabeled C2B8 -- such a strategy allows for utilization of the benefits of the immunologically active portion of C2B8 plus those benefits associated with a radiolabel. Preferred radiolabels include yttrium-90 given the larger circulating half-life of C2B8 versus the murine antibody 2B8. Because of the ability of C2B8 to deplete B-cells, and the benefits to be derived from the use of a radiolabel, a preferred alternative strategy is to treat the patient with C2B8 (either with a single dose or multiple doses) such that most, if not all, peripheral B cells have been depleted. This would then be followed with the use of radiolabeled 2B8; because of the depletion of peripheral B cells, the radiolabeled 2B8 stands an increased chance of targeting tumor cells. Iodine [131] labeled 2B8 is preferably utilized, given the types of results reported in the literature with this label (*see* Kaminski). An alternative preference involves the use of a radiolabeled 2B8 (or C2B8) first in an effort to increase the permeability of a tumor, followed by single or multiple treatments with C2B8; the intent of this strategy is to increase the chances of the C2B8 in getting both outside and inside the tumor mass. A further strategy involved the use of chemotherapeutic agent in combination with C2B8. These strategies include so-called "staggered" treatments, *ie*, treatment with chemotherapeutic agent, followed by treatment with C2B8, followed by a repetition of this protocol. Alternatively, initial treatment with a single or multiple doses of C2B8, thereafter followed with chemotherapeutic treatment, is viable. Preferred chemotherapeutic agents include, but are not limited to:



cyclophosphamide; doxorubicin; vincristine; and prednisone, *See Armitage, J.O. et al., Cancer 50:1695 (1982)*, incorporated herein by reference.

The foregoing alternative therapy strategies are not intended to be limiting, but  
5 rather are presented as being representative.

## VI. DEPOSIT INFORMATION

Anti-CD20 in TCAE 8 (transformed in *E. coli* for purposes of deposit) was  
10 deposited with the American Type Culture Collection (ATCC), 12301 Parklawn  
Drive, Rockville, Maryland, 20852, under the provisions of the Budapest Treaty  
for the International Recognition of the Deposit of Microorganisms for the  
Purpose of Patent Procedure ("Budapest Treaty"). The microorganism was  
tested by the ATCC on November 9, 1992, and determined to be viable on that  
15 date. The ATCC has assigned this microorganism for the following ATCC deposit  
number: ATCC 69119 (anti-CD20 in TCAE 8). Hybridoma 2B8 was deposited  
with the ATCC on June 22, 1993 under the provisions of the Budapest Treaty.  
The viability of the culture was determined on June 25, 1993 and the ATCC has  
assigned this hybridoma the following ATCC deposit number: HB 11388.

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## VII. In Vivo (Human) Y2B8 Dosimetry Studies

Forty-two patients with B-cell NHL were imaged following injection of either 100 or 250 mg/m<sup>2</sup> of unlabelled chimeric anti-CD20 monoclonal antibody (RITUXAN®) followed by 5 mg of mouse anti-CD20 labeled with 5mCi <sup>111</sup>In(IDEC-In2B8). The same doses were repeated in the first six patients at Day 7 to determine the first dose effect on the second (therapeutic) dose distribution. The patients had <sup>111</sup>In dosimetry performed by serial whole body  $\gamma$ -camera imaging, urine collection, and blood sampling at 0, 2, 6, 24, 48, 72, 96 and 144 hours. Organ activity was calculated at each time using the geometric mean technique. Images were corrected for attenuation by effective body thickness measurements from CT scans. The <sup>111</sup>In and <sup>90</sup>Y tumor, normal organ, and whole body remainder radiation doses were calculated using MIRDOSE3 (Oak Ridge Assoc. Univ.) based on times calculated using curve-fitting modeling. The highest mean calculated <sup>90</sup>Y radiation dose to a normal organ was the spleen (possibly tumor-containing) with 24.16 rads/mCi (range 0134 - 6697) followed by the liver with 17.17 rad/mCi (range 9.36 - 39.22) and lungs with 12.88 rad/mCi (range 4.2 - 67.71). Whole blood activity of <sup>90</sup>Y measured post-treatment and <sup>90</sup>Y predicted from IDEC-In2B8 administration was plotted against time for ten patients to compare the 2 decay curves. For each of these graphs, a monoexponential curve gave the best fits and T<sub>1/2</sub> were calculated. These data indicate that <sup>111</sup>In can operate as a predictor of <sup>90</sup>Y. Comparison was made with direct measurements of bone marrow biopsy specimens at 5-7 days post-

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dosing. Biopsies for seven patients were analyzed. Number of  $\mu\text{Ci}^{90}\text{Y}$  reaching the red marrow were estimated using two methods. The means were  $114\mu\text{Ci}$  and  $171.0\mu\text{Ci}$  by the two methods. These are below the MIRDSE3 calculated values and well below the estimated MTD for red marrow. The red marrow dose was calculated using  $^{90}\text{Y}$  whole blood  $\mu\text{Ci/gm}$  activity with the mean dose being  $5.85 \text{ rad/mCi}$  (range  $2.26 - 9.69$ ). The normal organ and lymphoma tumor radiation dose calculations for  $^{90}\text{Y}$  labeled anti-CD20 monoclonal antibody appear favorable when the "cold" antibody is used as a blocker and should allow therapeutic radiation doses to be given safely with the added therapeutic benefit of RITUXAN®.

#### VIII. In vivo (Human) Y2B8/C2B8 Combination Radiotherapy

IDEC-Y2B8 is a murine IgG<sub>1</sub> kappa monoclonal antibody MX-DTPA linked to the isotope  $^{90}\text{Y}$ ttrium and directed against the CD20 antigen. A 64% response rate had been previously demonstrated in a Phase I dose-escalating relapsed NHL trial requiring bone marrow harvest (Knox et al) and utilizing murine IDEC-2B8 as the cold clearing antibody. The current Phase I/II study used chimeric antibody RITUXAN® (IDEC-C2B8) as the unlabelled clearing antibody. The Group 1 segment of this study compared  $100 \text{ mg/m}^2$  with  $250 \text{ mg/m}^2$  of RITUXAN® as the clearing dose and compared dosimetry imaging capabilities. These patients did not receive Y2B8, but rather a therapeutic course of RITUXAN® ( $375 \text{ mg/m}^2$  for four doses) after imaging comparison was completed. It was determined that  $250 \text{ mg/m}^2$  was the optimum dose to be used in the Group 2 Phase I

segment of the study. Group 2 patients were dose-escalated from 0.2 mCi/kg to 0.4 mCi/kg Y2B8. No bone marrow or stem cell harvest was required. Interim analysis of 20 patients in whom full safety data is available indicates that 3 of 6 patients with baseline platelet counts between 100,000 and 150,000 developed transient Grade 4 hematologic toxicity whereas none of 14 patients treated at 0.2, 0.3 or 0.4 mCi/kg whose baseline platelet counts were >150,000 developed Grade 4 hematologic toxicity. Standard dose for the Phase II portion of the trial was chosen to be at 0.4 mCi/kg Y2B8. Patients with mild thrombocytopenia (platelets 100,000 - 150,000) were treated at a reduced dose of 0.3 mCi/kg. There were no other significant treatment-related toxicities and no patient developed HACA. Eighty-one percent (13/16 patients for whom response evaluation was available) of relapsed or refractory low-grade or follicular NHL patients responded to either 0.2, 0.3, or 0.4 mCi/kg in the Phase I or the Phase II portion of the trial. Forty-three percent (3/7 patients) of the intermediate-grade NHL and none (0.3) of the mantle cell patients responded in Groups 2 or 3. Single dose Y2B8 radioimmunotherapy has clinical activity and is well tolerated in patients with relapsed or refractory low-grade or follicular NHL and can be given safely in patients with mild thrombocytopenia.

SEQUENCE LISTING

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REFF, Mitchell E.  
RASTETTER, William H.
- (ii) TITLE OF INVENTION: THERAPEUTIC APPLICATION OF CHIMERIC AND  
RADIOLABELED ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED  
DIFFERENTIATION ANTIGEN FOR THE TREATMENT OF B CELL  
LYMPHOMA
- (iii) NUMBER OF SEQUENCES: 9
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- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: US 08/149,099  
(B) FILING DATE: 03-NOV-1993  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 07/978,891  
(B) FILING DATE: 12-NOV-1992
- (viii) ATTORNEY/AGENT INFORMATION:  
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAGCTTGG ATCGATCCTC TATGGTT

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8540 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GACGTCGCGG CCGCTCTAGG CCTCCAAAAA AGCCTCCTCA CTACTTCTGG AATAGCTCAG	60
AGGCCGAGGC GGCCTCGGCC TCTGCATAAA TAAAAAAAAT TAGTCAGCCA TGCATGGGGC	120
GGAGAATGGG CGGAAGTGGG CGGAGTTAGG GCGGGGATGG GCGGAGTTAG GGGCGGGACT	180
ATGGTTGCTG ACTAATTGAG ATGCATGCTT TGCATACTTC TGCCTGCTGG GGAGCCTGGG	240
GACTTTCCAC ACCTGGTTGC TGACTAATTG AGATGCATGC TTTGCATACT TCTGCCTGCT	300
GGGGAGCCTG GGGACTTTCC ACACCCTAAC TGACACACAT TCCACAGAAT TAATTTCCCT	360
AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC	420
GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG	480
ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA	540
TGGGTGGACT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA	600
AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCCG CCTGGCATTG TGCCAGTAC	660
ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC	720
ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA	780
TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG	840
GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA	900
CGGTGGGAGG TCTATATAAG CAGAGCTGGG TACGTGAACC GTCAGATCGC CTGGAGACGC	960
CATCACAGAT CTCTCACCAT GAGGGTCCCC GCTCAGCTCC TGGGGCTCCT GCTGCTCTGG	1020
CTCCAGGTG CACGATGTGA TGGTACCAAG GTGGAAATCA AACGTACGGT GGCTGCACCA	1080
TCTGTCTTCA TCTTCCCGCC ATCTGATGAG CAGTTGAAAT CTGGAAGTGC CTCTGTTGTG	1140
TGCCTGCTGA ATAACTTCTA TCCCAGAGAG GCCAAAGTAC AGTGGAAGGT GGATAACGCC	1200
CTCCAATCGG GTAACCTCCA GGAGAGTGTC ACAGAGCAGG ACAGCAAGGA CAGCACCTAC	1260
AGCCTCAGCA GCACCCTGAC GCTGAGCAAA GCAGACTACG AGAAACACAA AGTCTACGCC	1320
TGCGAAGTCA CCCATCAGGG CCTGAGCTCG CCCGTCACAA AGAGCTTCAA CAGGGGAGAG	1380
TGTTGAATTC AGATCCGTTA ACGGTTACCA ACTACCTAGA CTGGATTTCGT GACAACATGC	1440
GGCCGTGATA TCTACGTATG ATCAGCCTCG ACTGTGCCTT CTAGTTGCCA GCCATCTGTT	1500
GTTTGCCCCCT CCCCCGTGCC TTCCTTGACC CTGGAAGGTG CCACTCCAC TGTCTTTCC	1560

AGACAGTGT	CTCTGCACAG	ATAAGGACAA	ACATTATTCA	GAGGGAGTAC	CCAGAGCTGA	3660
GACTCCTAAG	CCAGTGAGTG	GCACAGCATT	CTAGGGAGAA	ATATGCTTGT	CATCACCAGAA	3720
GCCTGATTCC	GTAGAGCCAC	ACCTTGGTAA	GGGCCAATCT	GCTCACACAG	GATAGAGAGG	3780
GCAGGAGCCA	GGGCAGAGCA	TATAAGGTGA	GGTAGGATCA	GTTGCTCCTC	ACATTTGCTT	3840
CTGACATAGT	TGTGTTGGGA	GCTTGATAG	CTTGACAGC	TCAGGGCTGC	GATTTGCGC	3900
CAAACCTGAC	GGCAATCCTA	GCGTGAAGGC	TGGTAGGATT	TTATCCCCGC	TGCCATCATG	3960
GTTGACCAT	TGAACTGCAT	CGTCGCCGTG	TCCCAAATA	TGGGGATTGG	CAAGAACGGA	4020
GACCTACCCT	GGCCTCCGCT	CAGGAACGAG	TTCAAGTACT	TCCAAAGAAT	GACCACAACC	4080
TCTTCAGTGG	AAGGTAAACA	GAATCTGGTG	ATTATGGGTA	GGAAAACCTG	GTTCTCCATT	4140
CCTGAGAACA	ATCGACCTTT	AAAGGACAGA	ATTAATATAG	TTCTCAGTAG	AGAACTCAAA	4200
GAACCACCAC	GAGGAGCTCA	TTTTCTTGCC	AAAAGTTTGG	ATGATGCCTT	AAGACTTATT	4260
GAACAACCGG	AATTGGCAAG	TAAAGTAGAC	ATGGTTTGGG	TAGTCGGAGG	CAGTTCTGTT	4320
TACCAGGAAG	CCATGAATCA	ACCAGGCCAC	CTTAGACTCT	TTGTGACAAG	GATCATGCAG	4380
GAATTTGAAA	GTGACACGTT	TTTCCCAGAA	ATTGATTTGG	GGAAATATAA	ACTTCTCCCA	4440
GAATACCCAG	GCGTCCTCTC	TGAGGTCCAG	GAGGAAAAG	GCATCAAGTA	TAAGTTTGAA	4500
GTCTACGAGA	AGAAAGACTA	ACAGGAAGAT	GCTTTCAAGT	TCTCTGCTCC	CCTCCTAAAG	4560
TCATGCATTT	TTATAAGACC	ATGGGACTTT	TGCTGGCTTT	AGATCAGCCT	CGACTGTGCC	4620
TTCTAGTTGC	CAGCCATCTG	TTGTTTGCCC	CTCCCCCGTG	CCTTCCTTGA	CCCTGGAAGG	4680
TGCCACTCCC	ACTGTCCTTT	CCTAATAAAA	TGAGGAAATT	GCATCGCATT	GTCTGAGTAG	4740
GTGTCATTCT	ATTCTGGGGG	GTGGGGTGGG	GCAGGACAGC	AAGGGGGAGG	ATTGGGAAGA	4800
CAATAGCAGG	CATGCTGGGG	ATGCGGTGGG	CTCTATGGAA	CCAGCTGGGG	CTCGAGCTAC	4860
TAGCTTTGCT	TCTCAATTTT	TTATTTGCAT	AATGAGAAAA	AAAGGAAAAT	TAATTTTAAC	4920
ACCAATTCAG	TAGTTGATTG	AGCAAATGCG	TTGCCAAAAA	GGATGCTTTA	GAGACAGTGT	4980
TCTCTGCACA	GATAAGGACA	AACATTATTC	AGAGGGAGTA	CCCAGAGCTG	AGACTCCTAA	5040
GCCAGTGAGT	GGCACAGCAT	TCTAGGGAGA	AATATGCTTG	TCATCACCAG	AGCCTGATTC	5100
CGTAGAGCCA	CACCTTGCTA	AGGGCCAATC	TGCTCACACA	GGATAGAGAG	GGCAGGAGCC	5160
AGGGCAGAGC	ATATAAGGTG	AGGTAGGATC	AGTTGCTCCT	CACATTTGCT	TCTGACATAG	5220
TTGTGTTGGG	AGCTTGGATC	GATCCTCTAT	GGTTGAACAA	GATGGATTGC	ACGCAGGTTC	5280
TCCGGCCGCT	TGGGTGGAGA	GGCTATTCGG	CTATGACTGG	GCACAACAGA	CAATCGGCTG	5340
CTCTGATGCC	GCCGTGTTCC	GGCTGTCAGC	GCAGGGGCGC	CCGTTCTTTT	TTGTCAAGAC	5400
CGACCTGTCC	GGTGCCCTGA	ATGAACTGCA	GGACGAGGCA	GCGCGGCTAT	CGTGGCTGGC	5460
CACGACGGGC	GTTCTTTGCG	CAGCTGTGCT	CGACGTTGTC	ACTGAAGCGG	GAAGGGACTG	5520
GCTGCTATTG	GGCGAAGTGC	CGGGGCAGGA	TCTCCTGTCA	TCTCACCTTG	CTCCTGCCGA	5580
GAAAGTATCC	ATCATGGCTG	ATGCAATGCG	GCGGCTGCAT	ACGCTTGATC	CGGCTACCTG	5640

TAATAAAATG	AGGAAATTGC	ATCGCATTGT	CTGAGTAGGT	GTCATTCTAT	TCTGGGGGGT	1620
GGGGTGGGGC	AGGACAGCAA	GGGGGAGGAT	TGGGAAGACA	ATAGCAGGCA	TGCTGGGGAT	1680
GCGGTGGGCT	CTATGGAACC	AGCTGGGGCT	CGACAGCTAT	GCCAAGTACG	CCCCCTATTG	1740
ACGTCAATGA	CGGTAAATGG	CCCGCCTGGC	ATTATGCCCA	GTACATGACC	TTATGGGACT	1800
TTCCTACTTG	GCAGTACATC	TACGTATTAG	TCATCGCTAT	TACCATGGTG	ATGCGGTTTT	1860
GGCAGTACAT	CAATGGGCGT	GGATAGCGGT	TTGACTCACG	GGGATTTCCT	AGTCTCCACC	1920
CCATTGACGT	CAATGGGAGT	TTGTTTTTGGC	ACCAAAATCA	ACGGGACTTT	CCAAAATGTC	1980
GTAACAACTC	CGCCCCATTG	ACGCAAATGG	GCGGTAGGCG	TGTACGGTGG	GAGGTCTATA	2040
TAAGCAGAGC	TGGGTACGTC	CTCACATTCA	GTGATCAGCA	CTGAACACAG	ACCCGTCGAC	2100
ATGGGTTGGA	GCCTCATCTT	GCTCTTCCTT	GTCGCTGTTG	CTACGCGTGT	CGCTAGCACC	2160
AAGGGCCCAT	CGGTCTTCCC	CCTGGCACCC	TCCTCCAAGA	GCACCTCTGG	GGGCACAGCG	2220
GCCCTGGGCT	GCCTGGTCAA	GGACTACTTC	CCCGAACCGG	TGACGGTGTC	GTGGAACCTA	2280
GGCGCCCTGA	CCAGCGGCGT	GCACACCTTC	CCGGCTGTCC	TACAGTCCTC	AGGACTCTAC	2340
TCCCTCAGCA	GCGTGGTGAC	CGTGCCCTCC	AGCAGCTTGG	GCACCCAGAC	CTACATCTGC	2400
AACGTGAATC	ACAAGCCCAG	CAACACCAAG	GTGGACAAGA	AAGCAGAGCC	CAAATCTTGT	2460
GACAAAACTC	ACACATGCCC	ACCGTGCCCA	GCACCTGAAC	TCCTGGGGGG	ACCGTCAGTC	2520
TTCCTCTTCC	CCCCAAAACC	CAAGGACACC	CTCATGATCT	CCCGGACCCC	TGAGGTCACA	2580
TGCGTGGTGG	TGGACGTGAG	CCACGAAGAC	CCTGAGGTCA	AGTTCAACTG	GTACGTGGAC	2640
GCGTGGGAGG	TGCATAATGC	CAAGACAAAG	CCGCGGGAGG	AGCAGTACAA	CAGCACGTAC	2700
CGTGTGGTCA	GCGTCCTCAC	CGTCCTGCAC	CAGGACTGGC	TGAATGGCAA	GGACTACAAG	2760
TGCAAGGTCT	CCAACAAAGC	CCTCCCAGCC	CCCATCGAGA	AAACCATCTC	CAAAGCCAAA	2820
GGGCAGCCCC	GAGAACCACA	GGTGTACACC	CTGCCCCCAT	CCCGGGATGA	GCTGACCAGG	2880
AACCAGGTCA	GCCTGACCTG	CCTGGTCAAA	GGCTTCTATC	CCAGCGACAT	CGCCGTGGAG	2940
TGGGAGAGCA	ATGGGCAGCC	GGAGAACAAC	TACAAGACCA	CGCCTCCCGT	GCTGGACTCC	3000
GACGGCTCCT	TCTTCCTCTA	CAGCAAGCTC	ACCGTGGACA	AGAGCAGGTG	GCAGCAGGGG	3060
AACGTCTTCT	CATGCTCCGT	GATGCATGAG	GCTCTGCACA	ACCACTACAC	GCAGAAGAGC	3120
CTCTCCCTGT	CTCCGGGTAA	ATGAGGATCC	GTTAACGGTT	ACCAACTACC	TAGACTGGAT	3180
TCGTGACAAC	ATGCGGCCGT	GATATCTACG	TATGATCAGC	CTCGACTGTG	CCTTCTAGTT	3240
GCCAGCCATC	TGTTGTTTGC	CCCTCCCCCG	TGCCTTCCTT	GACCCTGGAA	GGTGCCACTC	3300
CCACTGTCCT	TTCCTAATAA	AATGAGGAAA	TTGCATCGCA	TTGTCTGAGT	AGGTGTCATT	3360
CTATTCTGGG	GGGTGGGGTG	GGGCAGGACA	GCAAGGGGGA	GGATTGGGAA	GACAATAGCA	3420
GGCATGCTGG	GGATGCGGTG	GGCTCTATGG	AACCAGCTGG	GGCTCGACAG	CGCTGGATCT	3480
CCCGATCCCC	AGCTTTGCTT	CTCAATTTCT	TATTTGCATA	ATGAGAAAAA	AAGGAAAATT	3540
AATTTTAACA	CCAATTCAGT	AGTTGATTGA	GCAAATGCGT	TGCCAAAAAG	GATGCTTTAG	3600



CCCATTTCGAC	CACCAAGCGA	AACATCGCAT	CGAGCGAGCA	CGTACTCGGA	TGGAAGCCGG	5700
TCTTGTTCGAT	CAGGATGATC	TGGACGAAGA	GCATCAGGGG	CTCGCGCCAG	CCGAAGTGT	5760
CGCCAGGCTC	AAGGCGCGCA	TGCCCCGACG	CGAGGATCTC	GTCGTGACCC	ATGGCGATGC	5820
CTGCTTGCCG	AATATCATGG	TGGAAAATGG	CCGCTTTTCT	GGATTCATCG	ACTGTGGCCG	5880
GCTGGGTGTG	GCGGACCGCT	ATCAGGACAT	AGCGTTGGCT	ACCCGTGATA	TTGCTGAAGA	5940
GCTTGGCGGC	GAATGGGCTG	ACCGCTTCCT	CGTGCTTTAC	GGTATCGCCG	CTTCCCGATT	6000
CGCAGCGCAT	CGCCTTCTAT	CGCCTTCTTG	ACGAGTTCTT	CTGAGCGGGA	CTCTGGGGTT	6060
CGAAATGACC	GACCAAGCGA	CGCCCAACCT	GCCATCACGA	GATTTTCGATT	CCACCGCCGC	6120
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GCGCGGGGAT	CTCATGCTGG	AGTTCTTCGC	CCACCCCAAC	TTGTTTATTG	CAGCTTATAA	6240
TGGTTACAAA	TAAAGCAATA	GCATCACAAA	TTTCACAAAT	AAAGCATTTT	TTTCACTGCA	6300
TTCTAGTTGT	GGTTTGTCCA	AACTCATCAA	TCTATCTTAT	CATGTCTGGA	TCGCGGCCGC	6360
GATCCCGTCG	AGAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCTGTGTG	AAATTGTTAT	6420
CCGCTCACAA	TTCCACACAA	CATACGAGCC	GGAGCATAAA	GTGTAAAGCC	TGGGGTGCCT	6480
AATGAGTGAG	CTAACTCACA	TTAATTGCGT	TGCGCTCACT	GCCCGCTTTC	CAGTCGGGAA	6540
ACCTGTCGTG	CCAGCTGCAT	TAATGAATCG	GCCAACGCGC	GGGAGAGGC	GGTTTGCCTA	6600
TTGGGCGCTC	TTCCGCTTCC	TCGCTCACTG	ACTCGCTGCG	CTCGGTTCGT	CGGCTGCGGC	6660
GAGCGGTATC	AGCTCACTCA	AAGGCGGTAA	TACGGTTATC	CACAGAATCA	GGGGATAACG	6720
CAGGAAAGAA	CATGTGAGCA	AAAGGCCAGC	AAAAGGCCAG	GAACCGTAAA	AAGGCCGCGT	6780
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GTCAGAGGTG	GCGAAACCCG	ACAGGACTAT	AAAGATACCA	GGCGTTTCCC	CCTGGAAGCT	6900
CCCTCGTGCG	CTCTCCTGTT	CCGACCCTGC	CGCTTACCGG	ATACCTGTCC	GCCTTTCTCC	6960
CTTCGGGAAG	CGTGGCGCTT	TCTCAATGCT	CACGCTGTAG	GTATCTCAGT	TCGGTGTAGG	7020
TCGTTGCTC	CAAGCTGGGC	TGTGTGCACG	AACCCCCCGT	TCAGCCCCGAC	CGCTGCGCCT	7080
TATCCGGTAA	CTATCGTCTT	GAGTCCAACC	CGGTAAGACA	CGACTTATCG	CCACTGGCAG	7140
CAGCCACTGG	TAACAGGATT	AGCAGAGCGA	GGTATGTAGG	CGGTGCTACA	GAGTTCTTGA	7200
AGTGGTGGCC	TAACTACGGC	TACACTAGAA	GGACAGTATT	TGGTATCTGC	GCTCTGCTGA	7260
AGCCAGTTAC	CTTCGGAAAA	AGAGTTGGTA	GCTCTTGATC	CGGCAAAACA	ACCACCGCTG	7320
GTAGCGGTGG	TTTTTTTTGTT	TGCAAGCAGC	AGATTACGCG	CAGAAAAAAA	GGATCTCAAG	7380
AAGATCCTTT	GATCTTTTCT	ACGGGGTCTG	ACGCTCAGTG	GAACGAAAAC	TCACGTTAAG	7440
GGATTTTGGT	CATGAGATTA	TCAAAAAGGA	TCTTCACCTA	GATCCTTTTA	AATTAAAAAT	7500
GAAGTTTTTAA	ATCAATCTAA	AGTATATATG	AGTAAACTTG	GTCTGACAGT	TACCAATGCT	7560
TAATCAGTGA	GGCACCTATC	TCAGCGATCT	GTCTATTTTC	TTCATCCATA	GTTGCCTGAC	7620
TCCCCGTCGT	GTAGATAACT	ACGATACGGG	AGGGCTTACC	ATCTGGCCCC	AGTGCTGCAA	7680

TGATACCGCG	AGACCCACGC	TCACCGGCTC	CAGATTTATC	AGCAATAAAC	CAGCCAGCCG	7740
GAAGGGCCGA	GCGCAGAAGT	GGTCCTGCAA	CTTTATCCGC	CTCCATCCAG	TCTATTAATT	7800
GTTGCCGGGA	AGCTAGAGTA	AGTAGTTCGC	CAGTTAATAG	TTGCGCAAC	GTTGTTGCCA	7860
TTGCTACAGG	CATCGTGGTG	TCACGCTCGT	CGTTTGGTAT	GGCTTCATTC	AGCTCCGGTT	7920
CCCAACGATC	AAGGCGAGTT	ACATGATCCC	CCATGTTGTG	CAAAAAAGCG	GTTAGCTCCT	7980
TCGGTCTCTC	GATCGTTGTC	AGAAGTAAGT	TGGCCGCAGT	GTTATCACTC	ATGGTTATGG	8040
CAGCACTGCA	TAATTCTCTT	ACTGTCATGC	CATCCGTAAG	ATGCTTTTCT	GTGACTGGTG	8100
AGTACTCAAC	CAAGTCATTC	TGAGAATAGT	GTATGCGGCG	ACCGAGTTGC	TCTTGCCCCG	8160
CGTCAATACG	GGATAATACC	GCGCCACATA	GCAGAACTTT	AAAAGTGCTC	ATCATTGGAA	8220
AACGTTCTTC	GGGGCGAAAA	CTCTCAAGGA	TCTTACCGCT	GTTGAGATCC	AGTTCGATGT	8280
AACCCACTCG	TGCACCCAAC	TGATCTTCAG	CATCTTTTAC	TTTCACCAGC	GTTTCTGGGT	8340
GAGCAAAAAC	AGGAAGGCAA	AATGCCGCAA	AAAAGGGAAT	AAGGGCGACA	CGGAAATGTT	8400
GAATACTCAT	ACTCTTCCTT	TTTCAATATT	ATTGAAGCAT	TTATCAGGGT	TATTGTCTCA	8460
TGAGCGGATA	CATATTTGAA	TGTATTTAGA	AAAATAAACA	AATAGGGGTT	CCGCGCACAT	8520
TTCCCCGAAA	AGTGCCACCT					8540

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 9209 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACGTCGCGG	CCGCTCTAGG	CCTCCAAAAA	AGCCTCCTCA	CTACTTCTGG	AATAGCTCAG	60
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GGAGAATGGG	CGGAACTGGG	CGGAGTTAGG	GGCGGGATGG	GCGGAGTTAG	GGGCGGGACT	180
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GACTTTCCAC	ACCTGGTTGC	TGACTAATTG	AGATGCATGC	TTTGCATACT	TCTGCCTGCT	300
GGGGAGCCTG	GGGACTTTCC	ACACCCTAAC	TGACACACAT	TCCACAGAAT	TAATTTCCCT	360
AGTTATTAAT	AGTAATCAAT	TACGGGGTCA	TTAGTTCATA	GCCCATATAT	GGAGTTCCGC	420
GTTACATAAC	TTACGGTAAA	TGGCCCGCCT	GGCTGACCGC	CCAACGACCC	CCGCCCATTG	480
ACGTCAATAA	TGACGTATGT	TCCCATAGTA	ACGCCAATAG	GGACTTTCCA	TTGACGTCAA	540
TGGGTGGACT	ATTTACGGTA	AACTGCCCAC	TTGGCAGTAC	ATCAAGTGTA	TCATATGCCA	600

AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCC CCTGGCATTG TGCCCAGTAC	660
ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC	720
ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT ACCGGTTTGA CTCACGCGGA	780
TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG	840
GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA	900
CGGTGGGAGG TCTATATAAG CAGAGCTGGG TACGTGAACC GTCAGATCGC CTGGAGACGC	960
CATCACAGAT CTCTCACTAT GGATTTTCAG GTGCAGATTA TCAGCTTCCT GCTAATCAGT	1020
GCTTCAGTCA TAATGTCCAG AGGACAAATT GTTCTCTCCC AGTCTCCAGC AATCCTGTCT	1080
GCATCTCCAG GGGAGAAGGT CACAATGACT TGCAGGGCCA GCTCAAGTGT AAGTTACATC	1140
CACTGGTTCC AGCAGAAGCC AGGATCCTCC CCCAAACCCT GGATTTATGC CACATCCAAC	1200
CTGGCTTCTG GAGTCCCTGT TCGCTTCAGT GGCAGTGGGT CTGGGACTTC TTA CTCTCTC	1260
ACAATCAGCA GAGTGGAGGC TGAAGATGCT GCCACTTATT ACTGCCAGCA GTGGACTAGT	1320
AACCCACCCA CGTTCGGAGG GGGGACCAAG CTGGAAATCA AACGTACGGT GGCTGCACCA	1380
TCTGTCTTCA TCTTCCCGCC ATCTGATGAG CAGTTGAAAT CTGGAAGTGC CTCTGTTGTG	1440
TGCCTGCTGA ATAACTTCTA TCCCAGAGAG GCCAAAGTAC AGTGGAAGGT GGATAACGCC	1500
CTCCAATCGG GTAAC TCCA GGAGAGTGTC ACAGAGCAGG ACAGCAAGGA CAGCACCTAC	1560
AGCCTCAGCA GCACCCTGAC GCTGAGCAAA GCAGACTACG AGAAACACAA AGTCTACGCC	1620
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GTTTGCCCCCT CCCCCGTGCC TTCCTTGACC CTGGAAGGTG CCACTCCAC TGTCCTTTCC	1860
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GCGGTGGGCT CTATGGAACC AGCTGGGGCT CGACAGCTAT GCCAAGTACG CCCCCTATTG	2040
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CCATTGACGT CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC	2280
GTAACAACCTC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA	2340
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TGCAAGGCTT CTGGCTACAC ATTTACCAGT TACAATATGC ACTGGGTAAA ACAGACACCT	2580
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GGCACAGCGG	CCCTGGGCTG	CCTGGTCAAG	GACTACTTCC	CCGAACCGGT	GACGGTGTCTG	2940
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GAGTACAAGT	GCAAGGTCTC	CAACAAAGCC	CTCCCAGCCC	CCATCGAGAA	AACCATCTCC	3480
AAAGCCAAAG	GGCAGCCCCG	AGAACCACAG	GTGTACACCC	TGCCCCCATC	CCGGGATGAG	3540
CTGACCAAGA	ACCAGGTCAG	CCTGACCTGC	CTGGTCAAAG	GCTTCTATCC	CAGCGACATC	3600
GCCGTGGAGT	GGGAGAGCAA	TGGGCAGCCG	GAGAACAAC	ACAAGACCAC	GCCTCCCGTG	3660
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CAGAAGAGCC	TCTCCCTGTC	TCCGGGTAAA	TGAGGATCCG	TTAACGGTTA	CCAACTACCT	3840
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GTGCCACTCC	CACTGTCCTT	TCCTAATAAA	ATGAGGAAAT	TGCATCGCAT	TGTCTGAGTA	4020
GGTGTCAATC	TATTCTGGGG	GGTGGGGTGG	GGCAGGACAG	CAAGGGGGAG	GATTGGGAAG	4080
ACAATAGCAG	GCATGCTGGG	GATGCGGTGG	GCTCTATGGA	ACCAGCTGGG	GCTCGACAGC	4140
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AGGAAAATTA	ATTTTAACAC	CAATTCAGTA	GTTGATTGAG	CAAATGCGTT	GCCAAAAAGG	4260
ATGCTTTAGA	GACAGTGTTT	TCTGCACAGA	TAAGGACAAA	CATTATTTCAG	AGGGAGTACC	4320
CAGAGCTGAG	ACTCCTAAGC	CAGTGAGTGG	CACAGCATTC	TAGGGAGAAA	TATGCTTGTC	4380
ATCACCGAAG	CCTGATTCCG	TAGAGCCACA	CCTTGGTAAAG	GGCCAATCTG	CTCACACAGG	4440
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CATTTGCTTC	TGACATAGTT	GTGTTGGGAG	CTTGGATAGC	TTGGACAGCT	CAGGGCTGCG	4560
ATTTGCGGCC	AAACTTGACG	GCAATCCTAG	CGTGAAGGCT	GGTAGGATTT	TATCCCCGCT	4620
GCCATCATGG	TTCGACCATT	GAAGTGCATC	GTGCGCGTGT	CCCAAAATAT	GGGGATTGGC	4680

AAGAACGGAG	ACCTACCCTG	GCCTCCGCTC	AGGAACGAGT	TCAAGTACTT	CCAAAGAATG	4740
ACCACAACCT	CTTCAGTGGA	AGGTAAACAG	AATCTGGTGA	TTATGGGTAG	GAAAACCTGG	4800
TTCTCCATTC	CTGAGAAGAA	TCGACCTTTA	AAGGACAGAA	TTAATATAGT	TCTCAGTAGA	4860
GAACTCAAAG	AACCACCACG	AGGAGCTCAT	TTTCTTGCCA	AAAGTTTGGA	TGATGCCTTA	4920
AGACTTATTG	AACAACCGGA	ATTGGCAAGT	AAAGTAGACA	TGGTTTGGAT	AGTCGGAGGC	4980
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CTTCTCCCAG	AATACCCAGG	CGTCCTCTCT	GAGGTCCAGG	AGGAAAAAGG	CATCAAGTAT	5160
AAGTTTGAAG	TCTACGAGAA	GAAAGACTAA	CAGGAAGATG	CTTTCAGTT	CTCTGCTCCC	5220
CTCCTAAAGC	TATGCATTTT	TATAAGACCA	TGGGACTTTT	GCTGGCTTTA	GATCAGCCTC	5280
GACTGTGCCT	TCTAGTTGCC	AGCCATCTGT	TGTTTGCCCC	TCCCCCGTGC	CTTCCTTGAC	5340
CCTGGAAGGT	GCCACTCCCA	CTGTCCTTTC	CTAATAAAAT	GAGGAAATTG	CATCGCATTG	5400
TCTGAGTAGG	TGTCATTCTA	TTCTGGGGGG	TGGGGTGGGG	CAGGACAGCA	AGGGGGAGGA	5460
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AATCGGCTGC	TCTGATGCCG	CCGTGTTCCG	GCTGTCAGCG	CAGGGGCGCC	CGGTTCTTTT	6060
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GGAAGCCGGT	CTTGTCGATC	AGGATGATCT	GGACGAAGAG	CATCAGGGGC	TCGCGCCAGC	6420
CGAACTGTTC	GCCAGGCTCA	AGGCGCGCAT	GCCCGACGGC	GAGGATCTCG	TCGTGACCCA	6480
TGGCGATGCC	TGCTTGCCGA	ATATCATGGT	GGAAAATGGC	CGCTTTTCTG	GATTCATCGA	6540
CTGTGGCCCG	CTGGGTGTGG	CGGACCGCTA	TCAGGACATA	GCGTTGGCTA	CCCGTGATAT	6600
TGCTGAAGAG	CTTGGCGGCG	AATGGGCTGA	CCGCTTCCTC	GTGCTTTACG	GTATCGCCGC	6660
TCCCGATTCT	CAGCGCATCG	CCTTCTATCG	CCTTCTTGAC	GAGTTCTTCT	GAGCGGGACT	6720

CTGGGGTTCG AAATGACCGA CCAAGCGACG CCCAACCTGC CATCACGAGA TTTCGATTCC	6780
ACCGCCGCCT TCTATGAAAG GTTGGGCTTC GGAATCGTTT TCCGGGACGC CGGCTGGATG	6840
ATCCTCCAGC GCGGGGATCT CATGCTGGAG TTCTTCGCCC ACCCCAATT GTTTATTGCA	6900
GCTTATAATG GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTTT	6960
TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATC TATCTTATCA TGTCTGGATC	7020
GCGGCCGCGA TCCCGTCGAG AGCTTGGCGT AATCATGGTC ATAGCTGTTT CCTGTGTGAA	7080
ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG AAGCATAAAG TGTAAGCCT	7140
GGGGTGCCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT GCGCTCACTG CCCGCTTTCC	7200
AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG	7260
GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTCGTTT	7320
GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG	7380
GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG AACCGTAAAA	7440
AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCC TGACGAGCAT CACAAAAATC	7500
GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC	7560
CTGGAAGCTC CCTCGTGCGC TCTCCTGTTT CGACCCGTC GCTTACCGGA TACCTGTCCG	7620
CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCAATGCTC ACGCTGTAGG TATCTCAGTT	7680
CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC	7740
GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAGACAC GACTTATCGC	7800
CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG GTATGTAGGC GGTGCTACAG	7860
AGTTCTTGAA GTGGTGGCCT AACTACGGCT AACTAGAAG GACAGTATTT GGTATCTGCG	7920
CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAAACAAA	7980
CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAG	8040
GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG AACGAAACT	8100
CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT CTTACCTAG ATCCTTTTAA	8160
ATTAAAAATG AAGTTTTTAA TCAATCTAAA GTATATATGA GTAAACTTGG TCTGACAGTT	8220
ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTTCTG TCATCCATAG	8280
TTGCCTGACT CCCCCTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA TCTGGCCCCA	8340
GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA GCAATAAACC	8400
AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT	8460
CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG	8520
TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA	8580
GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG	8640
TTAGCTCCTT CGGTCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGAGTG TTATCACTCA	8700
TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC ATCCGTAAGA TGCTTTTCTG	8760

TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT 8820  
 CTTGCCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA 8880  
 TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA 8940  
 GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT TTCACCAGCG 9000  
 TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC 9060  
 GGAAATGTTG AATACTCATA CTCTTCCTTT TTCAATATTA TTGAAGCATT TATCAGGGTT 9120  
 ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC 9180  
 CGCGCACATT TCCCCGAAAA GTGCCACCT 9209

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 47 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCACAGATC TCTCACCATG GATTTTCAGG TGCAGATTAT CAGCTTC

47

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGCAGCATCC GTACGTTTGA TTTCCAGCTT

30

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 384 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..384

(ix) FEATURE:

(A) NAME/KEY: mat\_peptide

(B) LOCATION: 67..384

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG GAT TTT CAG GTG CAG ATT ATC AGC TTC CTG CTA ATC AGT GCT TCA	48
Met Asp Phe Gln Val Gln Ile Ile Ser Phe Leu Leu Ile Ser Ala Ser	
-22 -20 -15 -10	
GTG ATA ATG TCC AGA GGG CAA ATT GTT CTC TCC CAG TCT CCA GCA ATC	96
Val Ile Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro Ala Ile	
-5 -1 1 5 10	
CTG TCT GCA TCT CCA GGG GAG AAG GTC ACA ATG ACT TGC AGG GCC AGC	144
Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala Ser	
15 20 25	
TCA AGT GTA AGT TAC ATC CAC TGG TTC CAG CAG AAG CCA GGA TCC TCC	192
Ser Ser Val Ser Tyr Ile His Trp Phe Gln Gln Lys Pro Gly Ser Ser	
30 35 40	
CCC AAA CCC TGG ATT TAT GCC ACA TCC AAC CTG GCT TCT GGA GTC CCT	240
Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro	
45 50 55	
GTT CGC TTC AGT GGC AGT GGG TCT GGG ACT TCT TAC TCT CTC ACA ATC	288
Val Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile	
60 65 70	
AGC AGA GTG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG	336
Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp	
75 80 85 90	
ACT AGT AAC CCA CCC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATC AAA	384
Thr Ser Asn Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys	
95 100 105	

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGCTCCCA CGCGTGTCTT GTCCAG

27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:



- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /note= "Nucleotide 3 is N wherein N is G or C."

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 18
- (D) OTHER INFORMATION: /note= "Nucleotide 18 is N wherein N is A or C."

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 19
- (D) OTHER INFORMATION: /note= "Nucleotide 19 is N wherein N is A or G."

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 25
- (D) OTHER INFORMATION: /note= "Nucleotide 25 is N wherein N is G or A."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGNTGTTGTG CTAGCTGNNG AGACNGTGA

29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 420 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..420

(ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 58..420

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG GGT TGG AGC CTC ATC TTG CTC TTC CTT GTC GCT GTT GCT ACG CGT	48
Met Gly Trp Ser Leu Ile Leu Leu Phe Leu Val Ala Val Ala Thr Arg	
-19 -15 -10 -5	
GTC CTG TCC CAG GTA CAA CTG CAG CAG CCT GGG GCT GAG CTG GTG AAG	96
Val Leu Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys	
-1 1 5 10	
CCT GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT TCT GGC TAC ACA TTT	144
Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe	
15 20 25	
ACC AGT TAC AAT ATG CAC TGG GTA AAA CAG ACA CCT GGT CGG GGC CTG	192
Thr Ser Tyr Asn Met His Trp Val Lys Gln Thr Pro Gly Arg Gly Leu	
30 35 40 45	
GAA TGG ATT GGA GCT ATT TAT CCC GGA AAT GGT GAT ACT TCC TAC AAT	240
Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn	
50 55 60	
CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA TCC TCC AGC	288
Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser	
65 70 75	
ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC	336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val	
80 85 90	
TAT TAC TGT GCA AGA TCG ACT TAC TAC GGC GGT GAC TGG TAC TTC AAT	384
Tyr Tyr Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr Phe Asn	
95 100 105	
GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC TCT GCA	420
Val Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ala	
110 115 120	

WHAT IS CLAIMED IS:

1. An improved method of treating B cell lymphoma which comprises administration of a therapeutic anti-CD20 antibody, wherein the improvement comprises administering at least one chemotherapeutic agent.
2. The method of Claim 1, wherein the antibody is a chimeric anti-CD20 antibody.
3. The method of Claim 1, wherein the chimeric antibody is C2B8.
4. The method of Claim 1, wherein the at least one chemotherapeutic agent is administered prior to antibody administration.
5. The method of Claim 1, wherein the at least one chemotherapeutic agent is administered simultaneous to antibody administration.
6. The method of Claim 1, wherein the at least one chemotherapeutic agent is administered after antibody administration.

7. The method of Claim 1, wherein the at least one chemotherapeutic agent is selected from the group consisting of cyclophosphamide, doxorubicin, vincristine, prednisone and mixtures thereof.

8. The method of Claim 7, wherein the at least one chemotherapeutic agent comprises a mixture of cyclophosphamide, doxorubicin, vincristine and prednisone, and the antibody is C2B8 or another anti-CD20 antibody that results in substantial depletion of peripheral B cells.

9. The method of Claim 8, wherein the antibody provides for nearly total peripheral blood B cell depletion within about 24 hours after administration.

10. The method of Claim 9, wherein the antibody dosage ranges from between about 0.4 and about 20 mg/kg body weight.





2/21

LINKER #1 15bp SV40 ORIGIN=332bp  
 GACGTCGCGG CCGCTCTAGG CCTCCAAAAA AGCCTCCTCA CTA CTCTTCTGG AATAGCTCAG 60  
 AGGCCGAGGC GGCCTCGGCC TCTGCATAAA TAAAAAAAT TAGTCAGCCA TGCATGGGGC 120  
 GGAGAATGGG CGGAACCTGGG CGGAGTTAGG GGCGGGATGG GCGGAGTTAG GGGCGGGACT 180  
 ATGGTTGCTG ACTAATTGAG ATGCATGCTT TGCATACTTC TGCTTGCTGG GGAGCCTGGG 240  
 GACTTTCCAC ACCTGGTTGC TGA CTAAATTG AGATGCATGC TTTGCATACT TCTGCCTGCT 300  
 GGGGAGCCTG GGGACTTTCC ACACCCTAAC TGACACACAT TCCACAGAAT TAATTCCCT 360  
 AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC 420  
 GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTTG 480  
 CMV PROMOTER-ENHANCER=567bp  
 ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA 540  
 TGGGTGGACT ATTTACGGTA AACTGCCAC TTTGGCAGTAC ATCAAGTGTA TCATATGCCA 600  
 AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCC CCTGGCATTG TGCCAGTAC 660  
 ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC 720  
 ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 780  
 TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG 840  
 GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA 900  
 CGGTGGGAGG TCTATATAAG CAGAGCTGGG TACCTGAACC GTCAGATCGC CTGGAGACGC 960  
 Bgl II CATCACAGAT CTCTCACCAT GAGGGTCCCC GCTCAGCTCC LEADER=60bp TGGGGCTCCT GCTGCTCTGG 1020  
 CTCCCAGGTG CACGATGTGA TGGTACCAAG GTGGAAATCA AACTGACGGT GGCTGCACCA 1080  
 TCTGTCTTCA TCTTCCCGCC ATCTGATGAG CAGTTGAAAT CTGGAAGTGC CTCTGTTGTG 1140  
 TGCTTGCTGA ATA ACTTCTA TCCAGAGAG GCCAAAGTAC AGTGGAAGGT GGATAACGCC 1200  
 HUMAN KAPPA CONSTANT 324bp 107 AMINO ACID & STOP CODON  
 CTCCAATCGG GTA ACTCCCA GGAGAGTGTC ACAGAGCAGG ACAGCAAGGA CAGCACCTAC 1260  
 AGCCTCAGCA GCACCCTGAC GCTGAGCAAA GCAGACTACG AGAAACACAA AGTCTACGCC 1320  
 TGCGAAGTCA CCCATCAGGG CCTGAGCTCG CCCGTCACAA AGAGCTTCAA CAGGGGAGAG 1380  
 STOP  
 LIGHT CHAIN Eco RI LINKER #4=85bp  
 TGTGTAATTC AGATCCGTTA ACGGTTACCA ACTACCTAGA CTGGATTCGT GACAACATGC 1440  
 GGCCGTGATA TCTACGTATG ATCAGCCTCG ACTGTGCCTT CTAGTTGCCA GCCATCTGTT 1500

FIG. 2A

3/21

GTTCCTTTCC 1560  
TAATAAAATG AGGAAATTGC ATCGCATTGT CTGAGTAGGT GTCATTCTAT TCTGGGGGGT 1620  
GGGGTGGGGC AGGACAGCAA GGGGGAGGAT TGGGAAGACA ATAGCAGGCA TGCTGGGGAT 1680  
GCGGTGGGCT CTATGGAACC AGCTGGGGCT CGACAGCTAT GCCAAGTACG CCCCTATTG 1740  
ACGTCAATGA CGGTAAATGG CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT 1800  
TTCCTACTTG GCAGTACATC TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT 1860  
GGCAGTACAT CAATGGGCGT GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC 1920  
CCATTGACGT CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CAAAATGTC 1980  
GTAACAACTC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA 2040  
TAAGCAGAGC TGGGTACGTC CTCACATTCA GTGATCAGCA CTGAACACAG ACCCGTCGAC 2100  
ATGGGTTGGA GCCTCATCTT GCTCTTCCTT GTCGCTGTTG CTACGCGTGT CGCTAGCACC 2160  
START HEAVY CHAIN  
AAGGGCCCAT CGGTCTTCCC CCTGGCACCC TCCTCCAAGA GCACCTCTGG GGGCACAGCG 2220  
GCCCTGGGCT GCCTGGTCAA GGACTIONC CCCGAACCGG TGACGGTGTC GTGGAACTCA 2280  
GGCGCCCTGA CCAGCGGCGT GCACACCTTC CCGGCTGTCC TACAGTCCTC AGGACTCTAC 2340  
TCCCTCAGCA GCGTGGTGAC CGTGCCCTCC AGCAGCTTGG GCACCCAGAC CTACATCTGC 2400  
AACGTGAATC ACAAGCCCAG CAACACCAAG GTGGACAAGA AAGCAGAGCC CAAATCTTGT 2460  
GACAAAACCTC ACACATGCCC ACCGTGCCCA GCACCTGAAC TCCTGGGGGG ACCGTCAGTC 2520  
TTCCTCTTCC CCCCCAAACC CAAGGACACC CTCATGATCT CCCGGACCCC TGAGGTCACA 2580  
TGCGTGGTGG TGGACGTGAG CCACGAAGAC CCTGAGGTCA AGTTCAACTG GTACGTGGAC 2640  
GGCGTGGAGG TGCATAATGC CAAGACAAAG CCGCGGGAGG AGCAGTACAA CAGCACGTAC 2700  
CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC CAGGACTGGC TGAATGGCAA GGACTIONAAG 2760  
TGCAAGGTCT CCAACAAAGC CCTCCCAGCC CCCATCGAGA AAACCATCTC CAAAGCCAAA 2820  
GGGCAGCCCC GAGAACCACA GGTGTACACC CTGCCCCCAT CCCGGGATGA GCTGACCAGG 2880  
AACCAGGTCA GCCTGACCTG CCTGGTCAAA GGCTTCTATC CCAGCGACAT CGCCGTGGAG 2940  
TGGGAGAGCA ATGGGCAGCC GGAGAACAAC TACAAGACCA CGCCTCCCGT GCTGGACTCC 3000

FIG. 2B



4/21

GACGGCTCCT TCTTCCTCTA CAGCAAGCTC ACCGTGGACA AGAGCAGGTG GCAGCAGGGG 3060  
AACGTCTTCT CATGCTCCGT GATGCATGAG GCTCTGCACA ACCACTACAC GCAGAAGAGC 3120  
**STOP HEAVY CHAIN** **Bam HI** **LINKER #7=81bp**  
CTCTCCCTGT CTCCGGGTAA ATGAGGATCC GTTAACGGTT ACCAACTACC TAGACTGGAT 3180  
3144 5  
TCGTGACAAC ATGCGGCCGT GATATCTACG TATGATCAGC CTCGACTGTG CTTTCTAGTT 3240  
3225 6  
GCCAGCCATC TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC 3300  
**BOVINE GROWTH HORMONE POLYADENYLATION REGION=231bp**  
CCACTGTCTT TCTTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGTCATT 3360  
CTATTCTGGG GGGTGGGGTG GGGCAGGACA GCAAGGGGGA GGATTGGGAA GACAATAGCA 3420  
GGCATGCTGG GGATGCGGTG GGCTCTATGG AACCAGCTGG GGCTCGACAG CGCTGGATCT 3480  
3456 7  
CCCGATCCCC AGCTTTGCTT CTCAATTTCT TATTTGCATA ATGAGAAAAA AAGGAAAATT 3540  
3490 1  
AATTTTAACA CCAATTCAGT AGTTGATTGA GCAAATGCGT TGCCAAAAAG GATGCTTTAG 3600  
**MOUSE BETA GLOBIN MAJOR PROMOTER=366bp**  
AGACAGTGTT CTCTGCACAG ATAAGGACAA ACATTATTCA GAGGGAGTAC CCAGAGCTGA 3660  
GACTCCTAAG CCAGTGAGTG GCACAGCATT CTAGGGAGAA ATATGCTTGT CATCACCGAA 3720  
GCCTGATTCC GTAGAGCCAC ACCTTGGTAA GGGCCAATCT GCTCACACAG GATAGAGAGG 3780  
GCAGGAGCCA GGGCAGAGCA TATAAGGTGA GGTAGGATCA GTTGCTCCTC ACATTTGCTT 3840  
CTGACATAGT TGTGTTGGGA **LINKER #9=19bp** GCTTGGATAG **5' UNTRANSLATED DHFR=82bp** CTTGACAGC TCAGGGCTGC GATTTGCGGC 3900  
3856 7 3875 6  
CAAACTTGAC GGCAATCCTA GCGTGAAGGC TGGTAGGATT TTATCCCCGC TGCCATCATG 3960  
3957 8  
GTTCGACCAT TGAAGTGCAT CGTCGCCGTG TCCCAAATA TGGGGATTGG CAAGAACGGA 4020  
GACCTACCCT GGCCTCCGCT CAGGAACGAG TTCAAGTACT TCCAAAGAAT GACCACAACC 4080  
TCTTCAGTGG AAGGTAAACA GAATCTGGTG ATTATGGGTA GGAAAACCTG GTTCTCCATT 4140  
**MOUSE DHFR=564bp=187 AMINO ACID & STOP CODON**  
CCTGAGAAGA ATCGACCTTT AAAGGACAGA ATTAATATAG TTCTCAGTAG AGAACTCAAA 4200  
GAACCACCAC GAGGAGCTCA TTTTCTTGCC AAAAGTTTGG ATGATGCCTT AAGACTTATT 4260  
GAACAACCGG AATTGGCAAG TAAAGTAGAC ATGGTTTGA TAGTCGGAGG CAGTTCTGTT 4320  
TACCAGGAAG CCATGAATCA ACCAGGCCAC CTTAGACTCT TTGTGACAAG GATCATGCAG 4380  
GAATTTGAAA GTGACACGTT TTTCCAGAA ATTGATTTGG GGAAATATAA ACTTCTCCCA 4440  
GAATACCCAG GCGTCCTCTC TGAGGTCCAG GAGGAAAAAG GCATCAAGTA TAAGTTTGAA 4500

FIG. 2C

5/21

STOP DHFR  
GTCTACGAGA AGAAAGAC TA ACAGGAAGAT GCTTTCAAGT TCTCTGCTCC CCTCCTAAAG 4560  
4521 2

3' UNTRANSLATED DHFR=82bp LINKER #10=10bp  
TCATGCATTT TTATAAGACC ATGGGACTTT TGCTGGCTTT AGATCAGCCT CGACTGTGCC 4620  
4603 4 4613 4

TTCTAGTTGC CAGCCATCTG TTGTTTGCCC CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG 4680

BOVINE GROWTH HORMONE POLYADENYLATION REGION=231bp  
TGCCACTCCC ACTGTCCTTT CCTAATAAAA TGAGGAAATT GCATCGCATT GTCTGAGTAG 4740

GTGTCATTCT ATTCTGGGGG GTGGGGTGGG GCAGGACAGC AAGGGGGAGG ATTGGGAAGA 4800

CAATAGCAGG CATGCTGGGG ATGCGGTGGG CTCTATGGAA CCAGCTGGGG CTGAGCTAC 4860  
4844 5

TAGCTTTGCT TCTCAATTC TTATTTGCAT AATGAGAAAA AAAGGAAAAT TAATTTTAAC 4920

ACCAATTCAG TAGTTGATTG AGCAAATGCG TTGCCAAAAA GGATGCTTTA GAGACAGTGT 4980

MOUSE BETA GLOBIN MAJOR PROMOTER=366bp  
TCTCTGCACA GATAAGGACA AACATTATTC AGAGGGAGTA CCCAGAGCTG AGACTCCTAA 5040

GCCAGTGAGT GGCACAGCAT TCTAGGGAGA AATATGCTTG TCATCACCGA AGCCTGATTC 5100

CGTAGAGCCA CACCTTGGTA AGGGCCAATC TGCTCACACA GGATAGAGAG GGCAGGAGCC 5160

AGGGCAGAGC ATATAAGGTG AGGTAGGATC AGTTGCTCCT CACATTTGCT TCTGACATAG 5220

LINKER #12=21bp START NEO  
TTGTGTTGGG AGCTTGGATC GATCCTCTAT GGTGAACAA GATGGATTGC ACGCAGGTTC 5280  
5227 8 5248 9

TCCGGCCGCT TGGGTGGAGA GGCTATTCCG CTATGACTGG GCACAACAGA CAATCGGCTG 5340

CTCTGATGCC GCCGTGTTCC GGCTGTCAGC GCAGGGGCGC CCGGTTCTTT TTGTCAAGAC 5400

NEOMYCIN PHOSPHOTRANSFERASE  
CGACCTGTCC GGTGCCCTGA ATGAAGTGA GGACGAGGCA GCGCGGCTAT CGTGGCTGGC 5460

795bp=264 AMINO ACIDS & STOP CODON  
CACGACGGGC GTTCCTTGCG CAGCTGTGCT CGACGTTGTC ACTGAAGCGG GAAGGGACTG 5520

GCTGCTATTG GGCGAAGTGC CGGGGCAGGA TCTCCTGTCA TCTACCTTG CTCCTGCCGA 5580

GAAAGTATCC ATCATGGCTG ATGCAATGCG GCGGCTGCAT ACGCTTGATC CGGCTACCTG 5640

CCCATTGAC CACCAAGCGA AACATCGCAT CGAGCGAGCA CGTACTCGGA TGGAAGCCGG 5700

TCTTGTCGAT CAGGATGATC TGGACGAAGA GCATCAGGGG CTCGCGCCAG CCGAACTGTT 5760

CGCCAGGCTC AAGGCGCGCA TGCCCGACGG CGAGGATCTC GTCGTGACCC ATGGCGATGC 5820

CTGCTTGCCG AATATCATGG TGGAAAATGG CCGCTTTTCT GGATTCATCG ACTGTGGCCG 5880

GCTGGGTGTG GCGGACCGCT ATCAGGACAT AGCGTTGGCT ACCCGTGATA TTGCTGAAGA 5940

GCTTGGCGGC GAATGGGCTG ACCGCTTCCT CGTGCTTTAC GGTATCGCCG CTTCCCGATTG 6000

FIG. 2D

6/21

GCAGCGCATC GCCTTCTATC GCCTTCTTGA CGAGTTCTTC <sup>STOP NEO</sup> TGAGCGGGGAC TCTGGGGTTC 6060  
6043 4

GAAATGACCG ACCAAGCGAC GCCCAACCTG CCATCACGAG ATTTTCGATTC CACCGCCGCC 6120

3' UNTRANSLATED NEO=173bp  
TTCTATGAAA GGTGTTGGGCTT CGGAATCGTT TTCCGGGACG CCGGCTGGAT GATCCTCCAG 6180

CGCGGGGATC TCATGCTGGA GTTCTTCGCC CACCCCAACT TGTTTATTGC AGCTTATAAT 6240  
6216 7

GGTTACAAAT AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT TCACTGCAT 6300

SV40 POLY A EARLY=133bp <sup>LINKER #13=19bp</sup>  
TCTAGTTGTG GTTTGTCCAA ACTCATCAAT CTATCTTATC ATGTCTGGAT CGCGGCCGCG 6360  
6349 50

ATCCCGTCTGA GAGCTTGGCG TAATCATGGT CATAGCTGTT TCCTGTGTGA AATTGTTATC 6420  
6368 9

CGCTCACAAAT TCCACACAAC ATACGAGCCG GAAGCATAAA GTGTAAAGCC TGGGGTGCCT 6480

AATGAGTGAG CTAACTCACA TTAATTGCGT TCGCTCACT GCCCGCTTTC CAGTCGGGAA 6540

ACCTGTCGTG CCAGCTGCAT TAATGAATCG GCCAACGCGC GGGGAGAGGC GGTTTTCGTA 6600

PVC 19  
TTGGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG CTCGGTCGTT CGGCTGCGGC 6660

GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG 6720

CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT 6780

6792=BACTERIAL ORIGIN OF REPLICATION  
TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA 6840

GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT 6900

CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC 6960

CTTCGGGAAG CGTGGCGCTT TCTCAATGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG 7020

TCGTTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT 7080

TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG 7140

CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA 7200

AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA 7260

AGCCAGTTAC CTTTCGAAAA AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG 7320

GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG CAGAAAAAAA GGATCTCAAG 7380

AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG 7440

GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT 7500

FIG. 2E

7/21

GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT 7560  
7550  
TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTTCG TTCATCCATA GTTGCTGAC 7620  
TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA 7680  
TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG 7740  
BETA LACTAMASE=861bp  
GAAGGGCCGA GCGCAGAAGT GGTCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT 7800  
286 AMINO ACID & STOP CODON  
GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC GTTGTTGCCA 7860  
TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT GGCTTCATTC AGCTCCGGTT 7920  
CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAGCG GTTAGCTCCT 7980  
TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG 8040  
CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG 8100  
AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGCCCGG 8160  
CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCATTGGAA 8220  
AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT 8280  
AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTACCAGC GTTTCTGGGT 8340  
GAGCAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT 8400  
START BETA LACTAMASE  
GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT TATTGTCTCA 8460  
8410  
TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT 8520  
TTCCCCGAAA AGTGCCACCT

FIG. 2F

8/21

LINKER #1=15bp  
GACGTCGCGG CCGCTCTAGG CCTCCAAAAA AGCCTCCTCA CTACTTCTGG AATAGCTCAG 60  
15 6

AGGCCGAGGC GGCCTCGGCC TCTGCATAAA TAAAAAAAT TAGTCAGCCA TGCATGGGGC 120

SV40 ORIGIN=332bp  
GGAGAATGGG CGGAAGTGGG CGGAGTTAGG GCGGGGATGG GCGGAGTTAG GGGCGGGACT 180  
ATGGTTGCTG ACTAATTGAG ATGCATGCTT TGCATACTTC TGCCTGCTGG GGAGCCTGGG 240  
GACTTTCCAC ACCTGGTTGC TGAATAATTG AGATGCATGC TTTGCATACT TCTGCCTGCT 300

LINKER #2=13bp  
GGGGAGCCTG GGGACTTTCC ACACCCTAAC TGACACACAT TCCACAGAAAT TAATTCCCCT 360  
347 8

AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC 420  
GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 480  
ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA 540

CVM PROMOTER-ENHANCER=567bp  
TGGGTGGACT ATTTACGGTA AACTGCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA 600  
AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTG TGCCAGTAC 660  
ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC 720  
ATGGTGATGC GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGG 780  
TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG 840  
GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA 900

LINKER #3=7bp  
CGGTGGGAGG TCTATATAAG CAGAGCTGGG TACGTGAACC GTCAGATCGC CTGGAGACGC 960  
927 8 934 5

Bgl 2 START LIGHT CHAIN NATURAL LEADER=66bp  
CATCACAGAT CTCTCACTAT GATTTCAG GTGCAGATTA TCAGCTTCCT GCTAATCAGT 1020  
978 9

GCTTCAGTCA TAATGTCCAG AGGACAAATT GTTCTCTCCC AGTCTCCAGC AATCCTGTCT 1080  
1044 5+1

GCATCTCCAG GGGAGAAGGT CACAATGACT TGCAGGGCCA GCTGAAGTGT AAGTTACATC 1140  
CACTGGTTCC AGCAGAAGCC AGGATCCTCC CCCAAACCCCT GGATTTATGC CACATCCAAC 1200

LIGHT CHAIN VARIABLE REGION 318bp 106 AMINO ACID  
CTGGCTTCTG GAGTCCCTGT TCGCTTCAGT GGCAGTGGGT CTGGGACTTC TTAATCTCTC 1260  
ACCATCAGCA GAGTGGAGGC TGAAGATGCT GCCACTTATT ACTGCCAGCA GTGGACTAGT 1320

BsiWI  
AACCCACCCA CGTTCGGAGG GGGGACCAAG CTGGAAATCA AACGTACGGT GGCTGCACCA 1380  
1362 3

TCTGTCTTCA TCTTCCCGCC ATCTGATGAG CAGTTGAAAT CTGGAACTGC CTCTGTTGTG 1440  
TGCCTGCTGA ATAATTCTA TCCAGAGAG GCCAAAGTAC AGTGAAGGT GGATAACGCC 1500

FIG. 3A

9/21

HUMAN KAPPA CONSTANT=324bp=107 AMINO ACID & STOP CODON  
 CTCCAATCGG GTAACCTCCA GGAGAGTGTC ACAGAGCAGG ACAGCAAGGA CAGCACCTAC 1560  
 AGCCTCAGCA GCACCCTGAC GCTGAGCAAA GCAGACTACG AGAAACACAA AGTCTACGCC 1620  
 TCGGAAGTCA CCCATCAGGG CCTGAGCTCG CCCGTCACAA AGAGCTTCAA CAGGGGAGAG 1680  
 STOP  
 LIGHT  
 CHAIN Eco RI LINKER #4=81bp  
 TGTGGAATTC AGATCCGTTA ACGGTTACCA ACTACCTAGA CTGGATTCTG GACAACATGC 1740  
 1646 7  
 GGCCGTGATA TCTACGTATG ATCAGCCTCG ACTGTGCCTT CTAGTTGCCA GCCATCTGTT 1800  
 1771 2  
 GTTTGCCCCCT CCCCCGTGCC TTCCTTGACC CTGGAAGGTG CCACTCCAC TGTCCTTTCC 1860  
 TAATAAAATG AGGAAATTGC ATCGCATTGT CTGAGTAGGT GTCATTCTAT TCTGGGGGGT 1920  
 BOVINE GROWTH HORMONE POLYADENYLATION REGION=231bp  
 GGGGTGGGGC AGGACAGCAA GGGGGAGGAT TGGGAAGACA ATAGCAGGCA TGCTGGGGAT 1980  
 GCGGTGGGCT CTATGGAACC AGCTGGGGCT CGACAGCTAT GCCAAGTACG CCCCTATTG 2040  
 2002 3 2017 8  
 ACGTCAATGA CGGTAAATGG CCCGCCTGGC ATTATGCCCA GTACATGACC TTATGGGACT 2100  
 TTCCTACTTG GCAGTACATC TACGTATTAG TCATCGCTAT TACCATGGTG ATGCGGTTTT 2160  
 CMV PROMOTER-ENHANCER=334bp  
 GGCAGTACAT CAATGGGCGT GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC 2220  
 CCATTGACGT CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CAAAATGTC 2280  
 GTAACAATC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG GAGGTCTATA 2340  
 LINKER #6=7bp  
 TAAGCAGAGC TGGGTACGTC CTCACATTCA GTGATCAGCA CTGAACACAG ACCCGTCGAC 2400  
 2351 2 2358 9  
 START  
 HEAVY CHAIN SYNTHETIC & NATURAL LEADER Mlu I 2457 8  
 ATGGGTTGGA GCCTCATCTT GCTCTTCCTT GTCGCTGTTG CTACGCGTGT CCTGTCCAG 2460  
 2401 -5 -4 -3 -2 -1 +1  
 GTACAACTGC AGCAGCCTGG GGCTGAGCTG GTGAAGCCTG GGGCCTCAGT GAAGATGTCC 2520  
 TGCAAGGCTT CTGGCTACAC ATTTACCAGT TACAATATGC ACTGGGTAAC ACAGACACCT 2580  
 HEAVY CHAIN VARIABLE=363bp=121 AMINO ACID  
 GGTGCGGGCC TGGAATGGAT TGGAGCTATT TATCCCGGAA ATGGTGATAC TTCCTACAAT 2640  
 CAGAAGTTCA AAGGCAAGGC CACATTGACT GCAGACAAAT CCTCCAGCAC AGCCTACATG 2700  
 CAGCTCAGCA GCCTGACATC TGAGGACTCT GCGGTCTATT ACTGTGCAAG ATCGACTTAC 2760  
 TACGGCGGTG ACTGGTACTT CAATGTCTGG GGCGCAGGGA CCACGGTCAC CGTCTCTGCA 2820  
 Nhe I  
 GCTAGCACCA AGGGCCCATC GGTCTTCCCC CTGGCACCCCT CCTCCAAGAG CACCTCTGGG 2880  
 GGCACAGCGG CCCTGGGCTG CCTGGTCAAG GACTACTTCC CCGAACCAGG GACGGTGTCTG 2940  
 HUMAN GAMMA 1 CONSTANT=993bp  
 TGGAACTCAG GCGCCCTGAC CAGCGGCGTG CACACCTTCC CGGCTGTCCT ACAGTCCTCA 3000

FIG. 3B

10/21

330 AMINO ACID & STOP CODON

GGACTCTACT CCCTCAGCAG CGTGGTGACC GTGCCCTCCA GCAGCTTGGG CACCCAGACC 3060  
TACATCTGCA ACGTGAATCA CAAGCCCAGC AACACCAAGG TGGACAAGAA AGCAGAGCCC 3120  
AAATCTTGTG AAAAACTCA CACATGCCCA CCGTGCCCAG CACCTGAACT CCTGGGGGGA 3180  
CCGTCACTCT TCCTCTTCCC CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT 3240  
GAGGTCACAT GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG 3300  
TACGTGGACG GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC 3360  
AGCACGTACC GTGTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG 3420  
GAGTACAAGT GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA AACCATCTCC 3480  
AAAGCCAAAG GGCAGCCCCG AGAACCACAG GTGTACACCC TGCCCCATC CCGGGATGAG 3540  
CTGACCAAGA ACCAGGTCAG CCTGACCTGC CTGGTCAAAG GCTTCTATCC CAGCGACATC 3600  
GCCGTGGAGT GGGAGAGCAA TGGGCAGCCG GAGAACAAC ACAAGACCAC GCCTCCCGTG 3660  
CTGGACTCCG ACGGCTCCTT CTTCTCTAC AGCAAGCTCA CCGTGGACAA GAGCAGGTGG 3720  
CAGCAGGGGA ACGTCTTCTC ATGCTCCGTG ATGCATGAGG CTCTGCACAA CCACTACACG 3780  
CAGAAGAGCC TCTCCCTGTC TCCGGGTAAA TGAGGATCCG TTAACGGTTA CCAACTACCT 3840  
STOP HEAVY CHAIN Bam HI LINKER #7=81bp  
3813 4  
AGACTGGATT CGTGACAACA TGCGGCCGTG ATATCTACGT ATGATCAGCC TCGACTGTGC 3900  
3894 5  
CTTCTAGTTG CCAGCCATCT GTTGTGTTGCC CCTCCCCCGT GCCTTCCTTG ACCCTGGAAG 3960  
GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT TGTCTGAGTA 4020  
GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG GATTGGGAAG 4080  
BOVINE GROWTH HORMONE POLYADENYLATION REGION=231bp  
ACAATAGCAG GCATGCTGGG GATGCGGTGG GCTCTATGGA ACCAGCTGGG GCTCGACAGC 4140  
4125 6  
GCTGGATCTC CCGATCCCCA GCTTTGCTTC TCAATTTCTT ATTTGCATAA TGAGAAAAAA 4200  
AGGAAAATTA ATTTTAACAC CAATTCAGTA GTTGATTGAG CAAATGCGTT GCCAAAAAGG 4260  
MOUSE BETA GLOBIN MAJOR PROMOTER=366bp  
ATGCTTTAGA GACAGTGGTC TCTGCACAGA TAAGGACAAA CATTATTGAG AGGGAGTACC 4320  
CAGAGCTGAG ACTCCTAAGC CAGTGAGTGG CACAGCATTC TAGGGAGAAA TATGCTTGTC 4380  
ATCACCGAAG CCTGATTCCG TAGAGCCACA CCTTGGTAAAG GGCCAATCTG CTCACACAGG 4440  
ATAGAGAGGG CAGGAGCCAG GGCAGAGCAT ATAAGGTGAG GTAGGATCAG TTGCTCCTCA 4500

FIG. 3C

11/21

CATTTGCTTC TGACATAGTT LINKER #9=19bp 5' UNTRANSLATED DHFR=82bp  
 4525 6 4544 5 4560  
 ATTTGCGGCC AAACCTTGACG GCAATCCTAG CGTGAAGGCT GGTAGGATTT TATCCCCGCT 4620  
START DHFR  
 GCCATCATGG TTCGACCATT GAACTGCATC GTCGCCGTGT CCCAAATAT GGGGATTGGC 4680  
 4626 7  
 AAGAACGGAG ACCTACCCTG GCCTCCGCTC AGGAACGAGT TCAAGTACTT CCAAAGAATG 4740  
 ACCACAACCT CTTCACTGGA AGGTAAACAG AATCTGGTGA TTATGGGTAG GAAAACCTGG 4800  
DHFR=564bp=187 AMINO ACID & STOP CODON  
 TTCTCCATTC CTGAGAAGAA TCGACCTTTA AAGGACAGAA TTAATATAGT TCTCAGTAGA 4860  
 GAACTCAAAG AACCACCACG AGGAGCTCAT TTTCTTGCCA AAAGTTTGGA TGATGCCTTA 4920  
 AGACTTATTG AACAACCGGA ATTGGCAAGT AAAGTAGACA TGGTTTGGAT AGTCGGAGGC 4980  
 AGTTCTGTTT ACCAGGAAGC CATGAATCAA CCAGGCCACC TTAGACTCTT TGTGACAAGG 5040  
 ATCATGCAGG AATTTGAAAG TGACACGTTT TTCCAGAAA TTGATTTGGG GAAATATAAA 5100  
 CTTCTCCCAG AATACCCAGG CGTCCTCTCT GAGGTCCAGG AGGAAAAAGG CATCAAGTAT 5160  
STOP DHFR 3' UNTRANSLATED DHFR=82bp  
 AAGTTTGAAG TCTACGAGAA GAAAGACTAA CAGGAAGATG CTTTCAAGTT CTCTGCTCCC 5220  
 5140 1  
 CTCCTAAAGC TATGCATTTT TATAAGACCA TGGGACTTTT GCTGGCTTTA LINKER #10  
=10bp GATCAGCCTC 5280  
 5272 3  
 GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTGTGCCCC TCCCCCGTGC CTTCCCTTGAC 5340  
BOVINE GROWTH HORMONE POLYADENYLATION=231bp  
 CCTGGAAGGT GCCACTCCCA CTGTCCCTTC CTAATAAAAT GAGGAAATTG CATCGCATTG 5400  
 TCTGAGTAGG TGTCATTCTA TTCTGGGGGG TGGGGTGGGG CAGGACAGCA AGGGGGAGGA 5460  
LINKER #11  
 TTGGGAAGAC AATAGCAGGC ATGCTGGGGA TCGGGTGGGC TCTATGGAAC CAGCTGGGGC 5520  
 5513 4  
=17bp  
 TCGAGCTACT AGCTTTGCTT CTCAATTTCT TATTTGCATA ATGAGAAAAA AAGGAAAATT 5580  
 5530 1  
 AATTTTAAAC CCAATTCAGT AGTTGATTGA GCAAATGCGT TGCCAAAAAG GATGCTTTAG 5640  
MOUSE BETA GLOBIN MAJOR PROMOTER=366bp  
 AGACAGTGTT CTCTGCACAG ATAAGGACAA CTAGGGAGAA ATATGCTTGT CATCACCGAA 5700  
 GACTCCTAAG CCAGTGAGTG GCACAGCATT CTAGGGAGAA ATATGCTTGT CATCACCGAA 5760  
 GCCTGATTCC GTAGAGCCAC ACCTTGGTAA GGGCCAATCT GCTCACACAG GATAGAGAGG 5820  
 GCAGGAGCCA GGGCAGAGCA TATAAGGTGA GGTAGGATCA GTTGCTCCTC ACATTTGCTT 5880  
LINKER #12=21bp START NEO  
 CTGACATAGT TGTGTTGGGA GCTTGGATCG ATCCTCTATG GTTGAACAAG ATGGATTGCA 5940  
 5896 7 5917 8  
 CGCAGGTTCT CCGGCCGCTT GGGTGGAGAG GCTATTCGGC TATGACTGGG CACAACAGAC 6000

FIG. 3D



12/21

AATCGGCTGC TCTGATGCCG CCGTGTTCCG GCTGTCAGCG CAGGGGCGTC CGGTTCTTTT 6060  
NEOMYCIN PHOSPHOTRANSFERASE=795bp=264 AMINO ACID & STOP CODON  
TGTCAGAGACC GACCTGTCCG GTGCCCTGAA TGAAGTGCAG GACGAGGCAG CGCGGCTATC 6120  
GTGGCTGGCC ACGACGGGCG TTCCTTGCGC AGCTGTGCTC GACGTTGTCA CTGAAGCGGG 6180  
AAGGGACTGG CTGCTATTGG GCGAAGTGCC GGGGCAGGAT CTCCTGTCAT CTCACCTTGC 6240  
TCCTGCCGAG AAAGTATCCA TCATGGCTGA TGCAATGCCG CGGCTGCATA CGCTTGATCC 6300  
GGCTACCTGC CCATTGACCC ACCAAGCGAA ACATCGCATC GAGCGAGCAC GTACTCGGAT 6360  
GGAAGCCGGT CTTGTGATC AGGATGATCT GGACGAAGAG CATCAGGGGC TCGCGCCAGC 6420  
CGAACTGTTC GCCAGGCTCA AGGCGCGCAT GCCCGACGGC GAGGATCTCG TCGTGACCCA 6480  
TGGCGATGCC TGCTTGCCGA ATATCATGGT GGAAAATGGC CGCTTTTCTG GATTCATCGA 6540  
CTGTGGCCGG CTGGGTGTGG CGGACCGCTA TCAGGACATA GCGTTGGCTA CCCGTGATAT 6600  
TGCTGAAGAG CTTGGCGGCG AATGGGCTGA CCGCTTCCTC GTGCTTTACG GTATCGCCGC 6660  
TCCCGATTCC CAGCGCATCG CTTCTATCG CTTCTTGAC GAGTTCTTCT GAGCGGGACT 6720  
STOP NEO  
6712 3  
CTGGGGTTCC AAATGACCGA CCAAGCGACG CCCAACCTGC CATCACGAGA TTTGATTCC 6780  
3' UNTRANSLATED NEO=173bp  
ACCGCCGCCT TCTATGAAAG GTTGGGCTTC GGAATCGTTT TCCGGGACGC CGGCTGGATG 6840  
ATCCTCCAGC GCGGGGATCT CATGCTGGAG TTCTTCGCC ACCCCAACTT GTTTATTGCA 6900  
6885 6  
GCTTATAATG GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT 6960  
SV40 EARLY POLYADENYLATION REGION=133bp  
TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATC TATCTTATCA TGTCTGGATC 7020  
7018 9  
LINKER #13=19bp  
GCGGCCGCGA TCCCGTCCGAG AGCTTGGCGT AATCATGGTC ATAGCTGTTT CCTGTGTGAA 7080  
7037 8  
PUC 19  
ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG AAGCATAAAG TGTAAGCCT 7140  
GGGGTGCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT GCGCTCACTG CCCGCTTTCC 7200  
AGTCGGGAAA CCTGTCGTGC CAGCTGCATT AATGAATCG CCAACGCGCG GGGAGAGGCG 7260  
GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTGCTTC 7320  
GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG 7380  
GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG AACCGTAAAA 7440  
7461=BACTERIAL ORIGIN OF REPLICATION  
AGGCCGCGTT GCTGGCGTTT TCCATAGGC TCCGCCCCC TGACGAGCAT CACAAAAATC 7500

FIG. 3E

13/21

GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC 7560  
 CTGGAAGCTC CCTCGTGCGC TCTCCTGTTT CGACCCCTGCC GCTTACCGGA TACCTGTCCG 7620  
 CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCAATGCTC ACGCTGTAGG TATCTCAGTT 7680  
 CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC 7740  
 GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAGACAC GACTTATCGC 7800  
 CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG GTATGTAGGC GGTGCTACAG 7860  
 AGTTCTTGAA GTGGTGGCCT AACTACGGCT AACTAGAAG GACAGTATTT GGTATCTGCG 7920  
 CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAACAAA 7980  
 CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAG 8040  
 GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG AACGAAAAC 8100  
 CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT CTTCACCTAG ATCCTTTTAA 8160  
 ATTAAAAATG AAGTTTTTAA TCAATCTAAA GTATATATGA GTAAACTTGG TCTGACAGTT STOP 8220  
BETA LACTAMASE  
 ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTCTG TCATCCATAG 8280  
 TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA TCTGGCCCCA 8340  
 GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA GCAATAAACC 8400  
 BETA LACTAMASE=861bp=286 AMINO ACID & STOP CODON  
 AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT 8460  
 CTATTAATTG TTGCCGGGAA GCTAGAGTAA GTAGTTCCGC AGTTAATAGT TTGCGCAACG 8520  
 TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA 8580  
 GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG 8640  
 TTAGCTCCTT CGGTCTCTCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG TTATCACTCA 8700  
 TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC ATCCGTAAGA TGCTTTTCTG 8760  
 TGACTGGTGA GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT 8820  
 CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA 8880  
 TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA 8940  
 GGTGATGTA ACCCACTCGT GCACCCAAC 8900  
 TTTCTGGGTG AGCAAAAACA GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC 9060  
 GGAAATGTTG AATACTCAT START BETA LACTAMASE CTCTTCTTTT TTCAATATTA TTGAAGCATT TATCAGGGTT 9120  
 ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC 9180  
 CGCGCACATT TCCCCGAAAA GTGCCACCT

FIG. 3F

LEADER

FRAME 1	Met	Asp	Phe	Gln	Val	Gln	Ile	Ile	Ser	Phe	Leu	Leu	Ile	Ser	Ala	Ser	Val		
	ATG	GAT	TTT	CAG	GTG	CAG	ATT	ATC	AGC	TTC	CTG	CTA	ATC	AGT	GCT	TCA	GTC		
			987			996			1005			1014			1023				
-5	Ile	Met	Ser	Arg	Gly	Gln	Ile	Val	Leu	Ser	Gln	Ser	Pro	Ala	Ile	Leu	Ser	Ala	Ser
	ATA	ATG	TCC	AGA	GGA	CAA	ATT	GTT	CTC	TCC	CAG	TCT	CCA	GCA	ATC	CTG	TCT	GCA	TCT
	1038				1047			1056			1065			1074			1083		
Pro	Gly	Glu	Lys	Val	Thr	Met	Thr	Cys	Arg	Ala	Ser	Ser	Ser	Val	Ser	Tyr	Ile	His	
CCA	GGG	GAG	AAG	GTC	ACA	ATG	ACT	TGC	AGG	GCC	AGC	TCA	AGT	GTA	AGT	TAC	ATC	CAC	
	1095			1104				1113			1122			1131			1140		
35		FR2			40			45					49	50		CDR2			
Trp	Phe	Gln	Gln	Lys	Pro	Gly	Ser	Ser	Pro	Lys	Pro	Trp	Ile	Tyr	Ala	Thr	Ser	Asn	
TGG	TTC	CAG	CAG	AAG	CCA	GGA	TCC	TCC	CCC	AAA	CCC	TGG	ATT	TAT	GCC	ACA	TCC	AAC	
	1152			1161				1170			1179			1188			1197		
55	56	57			60			FR3		65						70			
Leu	Ala	Ser	Gly	Val	Pro	Val	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Ser	Tyr	Ser	
CTG	GCT	TCT	GGA	GTC	CCT	GTT	CGC	TTC	AGT	GGC	AGT	GGG	TCT	GGG	ACT	TCT	TAC	TCT	
	1209			1218				1227		1236			1245			1254			
	75				80					85				88	89	90			
Leu	Thr	Ile	Ser	Arg	Val	Glu	Ala	Glu	Asp	Ala	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Trp	
CTC	ACC	ATC	AGC	AGA	GTG	GAG	GCT	GAA	GAT	GCT	GCC	ACT	TAT	TAC	TGC	CAG	CAG	TGG	
	1266			1275				1284		1293			1302			1311			
		CDR3	95		97	98		100	FR4			105		107					
Thr	Ser	Asn	Pro	Pro	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys				
ACT	AGT	AAC	CCA	CCC	ACG	TTC	GGA	GGG	GGG	ACC	AAG	CTG	GAA	ATC	AAA				
	1323			1332				1341		1350			1359						

FIG. 4

15/21

LEADER

-19		-15		-10		-5
FRAME 1	Met Gly Trp Ser Leu Ile Leu Leu Phe Leu Val Ala Val Ala Thr Arg Val					
	ATG GGT TGG AGC CTC ATC TTG CTC TTC CTT GTC GCT GTT GCT ACG CGT GTC					
	2409 2418 2427 2436 2445					
-1	+1	FR1		10		15
Leu Ser	Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Ala Gly Ala Ser					
CTG TCC	CAG GTA CAA CTG CAG CAG CCT GGG GCT GAG CTG GTG AAG CCT GGG GCC TCA					
	2460 2469 2478 2487 2496 2505					
20		25		30	31	CDR1
Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His Trp						
GTG AAG ATG TCC TGC AAG GCT TCT GGC TAC ACA TTT ACC AGT TAC AAT ATG CAC TGG						
	2517 2526 2536 2544 2553 2562					
40	FR2	45		49	50	52 52A 53 54
Val Lys Gln Thr Pro Gly Arg Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn						
GTA AAA CAG ACA CCT GGT CGG GGC CTG GAA TGG ATT GGA GCT ATT TAT CCC GGA AAT						
	2574 2583 2592 2601 2610 2615					
55	CDR2	60		65	66	FR3
Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys						
GGT GAT ACT TCC TAC AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT GCA GAC AAA						
	2631 2640 2649 2658 2667 2676					
75		80	82	82A 82B 82C 83		85
Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val						
TCC TCC AGC ACA GCC TAC ATG CAG CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC						
	2688 2697 2706 2715 2724 2733					
90		94 95	CDR3	100	100A 100B 100C 100D 101	102 103
Tyr Tyr Cys Ala Arg Ser Thr Tyr Tyr Gly Gly Asp Trp Tyr Phe Asn Val Trp Gly						
TAT TAC TGT GCA AGA TCG ACT TAC TAC GGC GGT GAC TGG TAC TTC AAT GTC TGG GGC						
	2745 2754 2763 2772 2781 2790					
105	FR4	110		113		
Ala Gly Thr Thr Val Thr Val Ser Ala						
GCA GGG ACC ACG GTC ACC GTC TCT GCA						
	2802 2811 2820					

FIG. 5

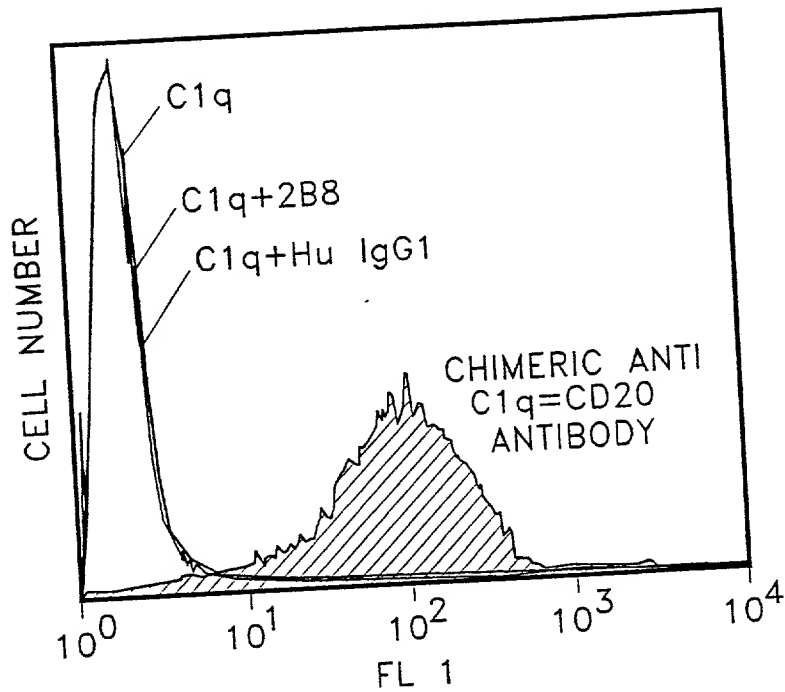


FIG. 6

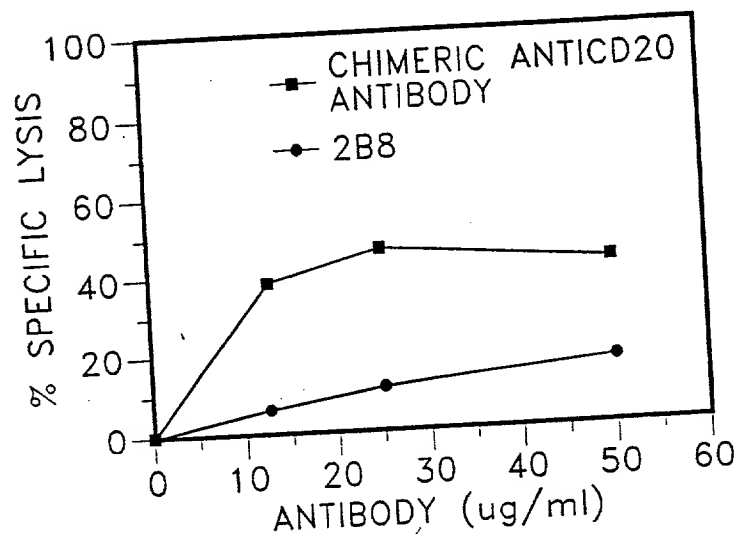


FIG. 7

17/21

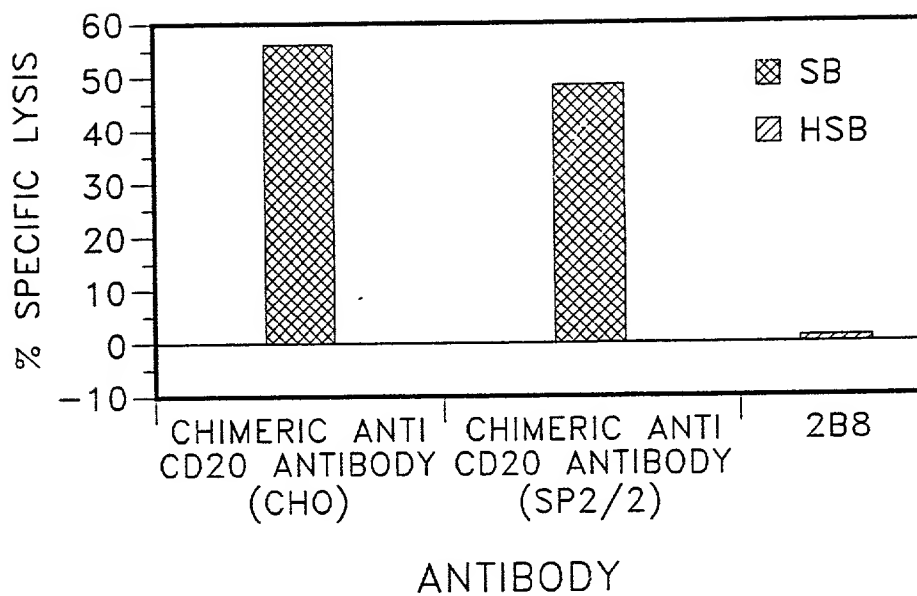


FIG. 8

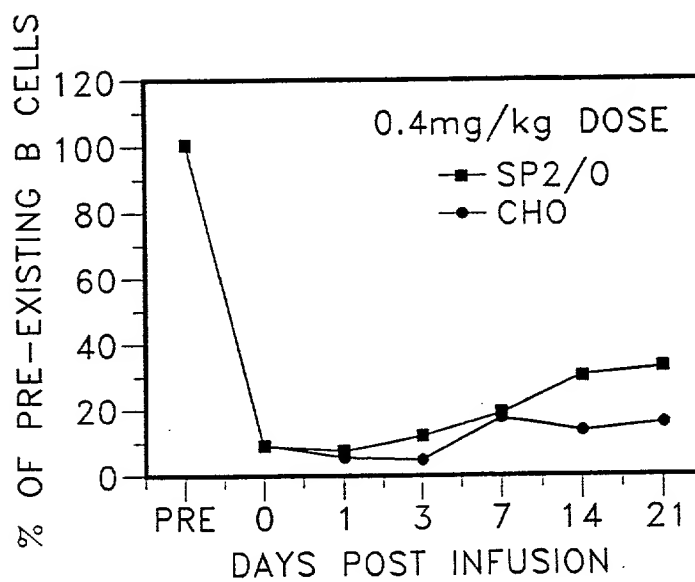
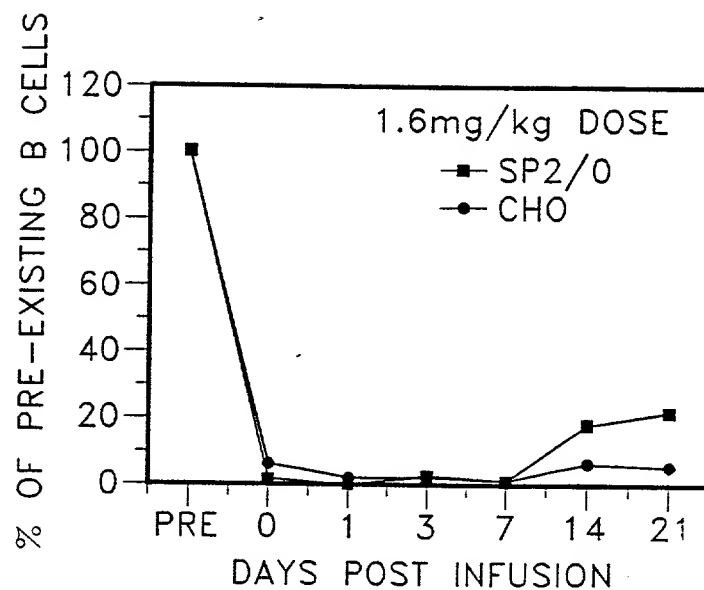
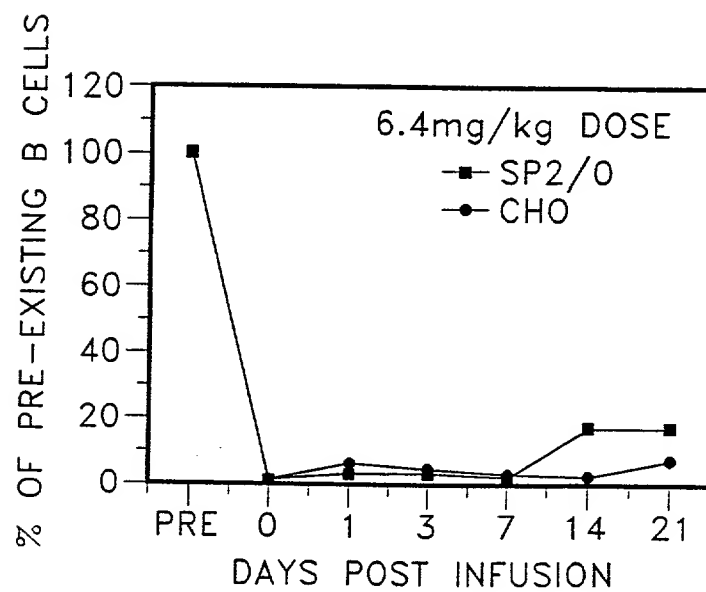
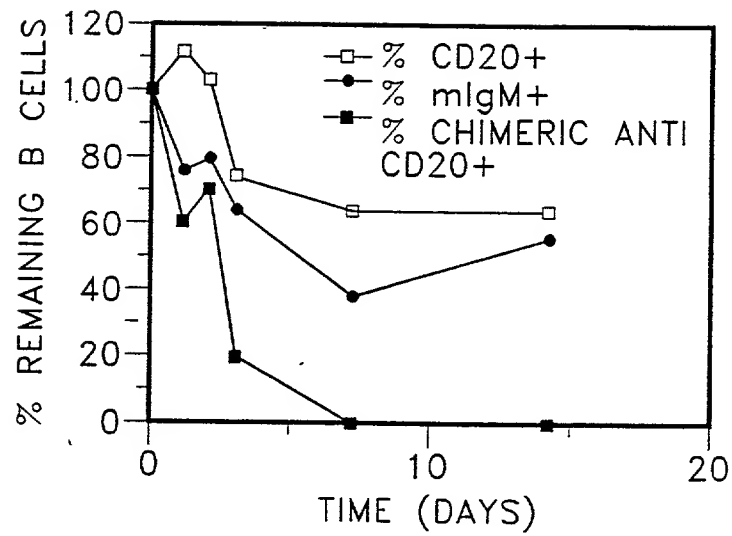
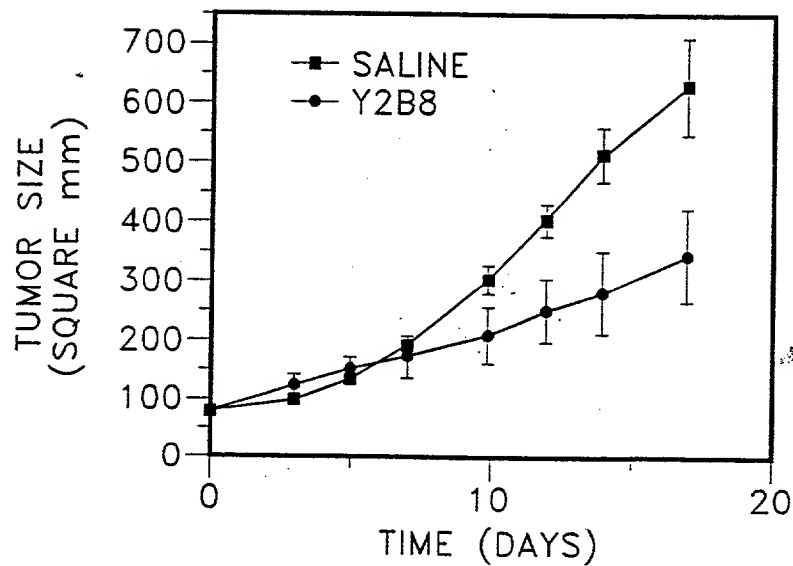
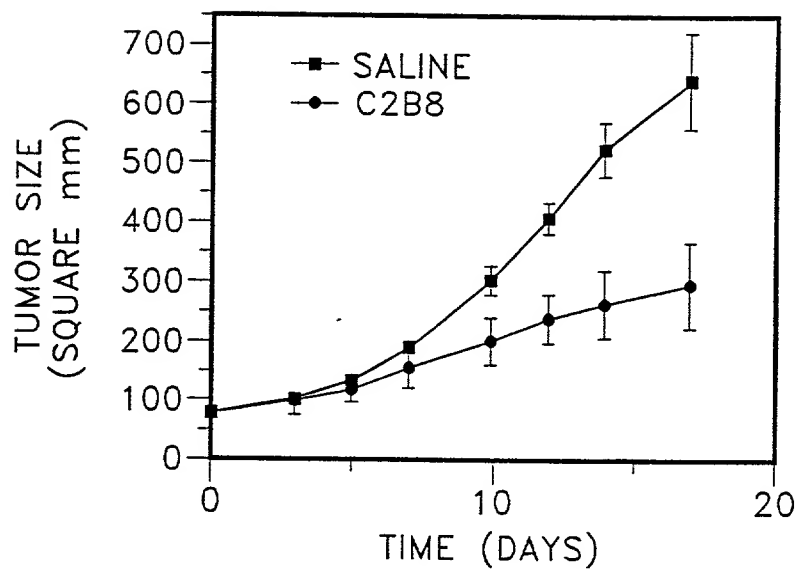
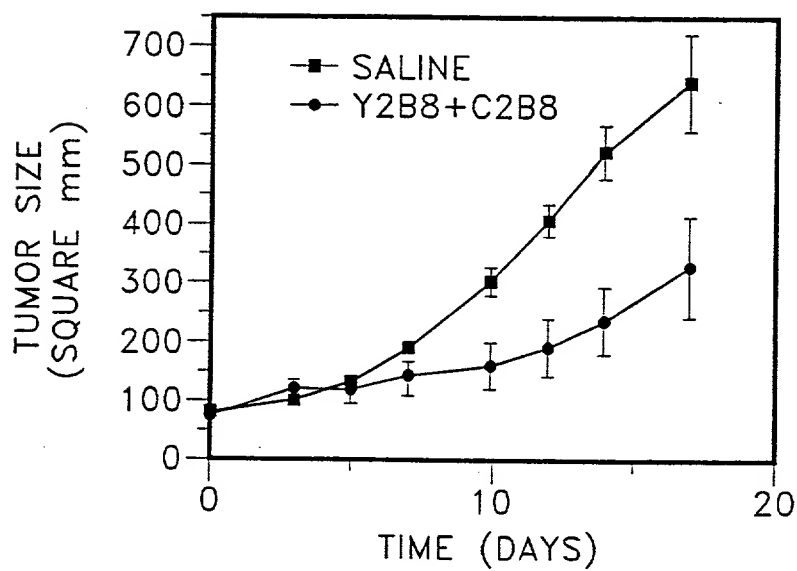


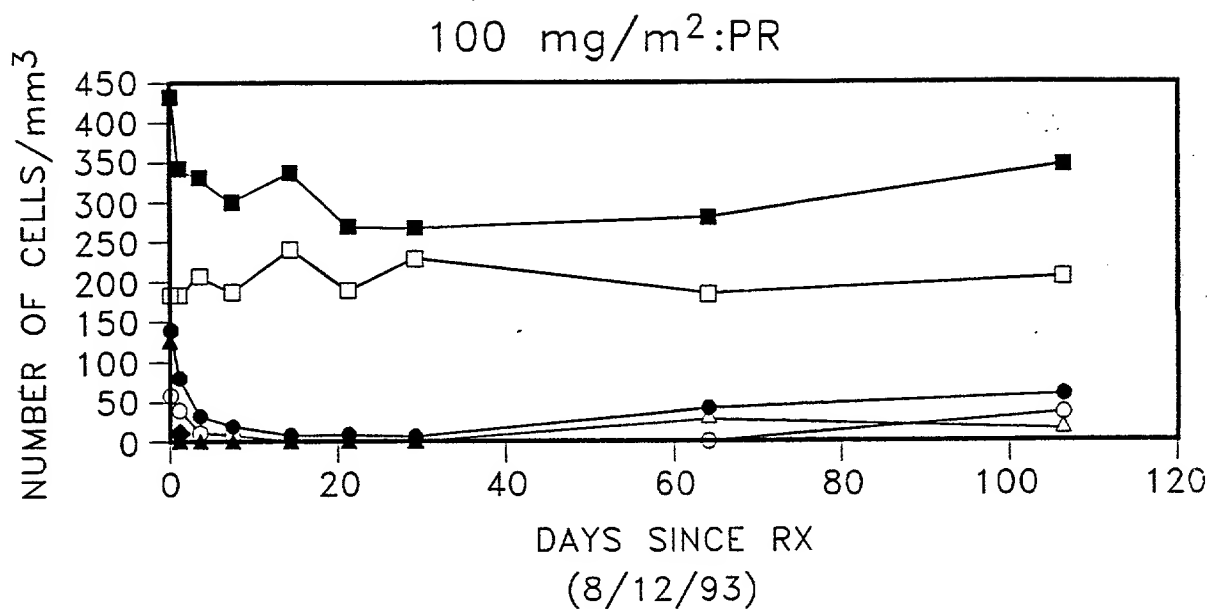
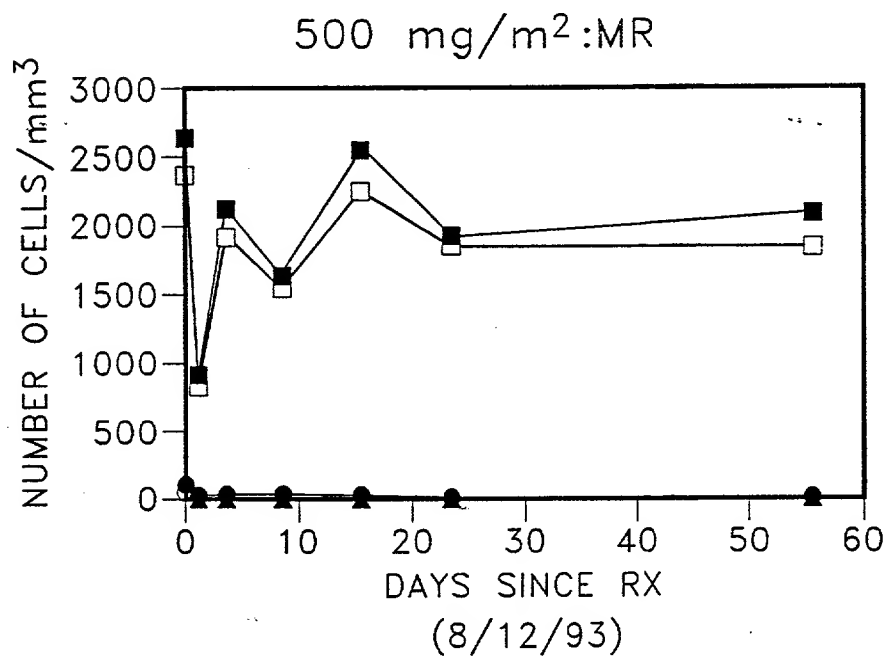
FIG. 9A

*FIG. 9B**FIG. 9C*

*FIG. 10**FIG. 11*



*FIG. 12**FIG. 13*

*FIG. 14A**FIG. 14B*

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of ) **Box PATENT APPLICATION**  
)  
Darrell R. ANDERSON et al ) Group Art Unit: Unassigned  
)  
Application No.: Unassigned ) Examiner: Unassigned  
)  
Filed: August 29, 1997 )  
)  
For: THERAPEUTIC APPLICATION OF )  
CHIMERIC AND RADIOLABELED )  
ANTIBODIES TO HUMAN B )  
LYMPHOCYTE RESTRICTED )  
DIFFERENTIATION ANTIGEN )  
FOR TREATMENT OF B CELL )  
LYMPHOMA )

**INFORMATION SHEET**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

As the above-identified application is being filed without an executed Declaration  
and Power of Attorney, the following information is provided:

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If any questions arise in connection with this application, kindly contact the  
undersigned attorney at the telephone number listed below.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: Robin L. Teskin  
Robin L. Teskin  
Registration No. 35,030

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Date: August 29, 1997

Applicant or Patentee: Darrell R. ANDERSON et al  
Application or Patent No.: Unassigned  
Filed or Issued: August 29, 1997  
For: THERAPEUTIC APPLICATION OF CHIMERIC AND RADIOLABELED ANTIBODIES TO HUMAN  
B LYMPHOCYTE RESTRICTED DIFFERENTIATION ANTIGEN FOR TREATMENT OF B CELL  
LYMPHOMA

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:  
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN IDEC Pharmaceutical Corporation  
ADDRESS OF CONCERN 11011 Torreyana Road  
San Diego, CA 92121

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.12, and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average, over the previous fiscal year of the concern, of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled THERAPEUTIC APPLICATION OF CHIMERIC AND RADIOLABELED ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED DIFFERENTIATION ANTIGEN FOR TREATMENT OF B CELL LYMPHOMA by inventor(s) Darrell R. ANDERSON, Nabil HANNA, John E. LEONARD, Roland A. NEWMAN, Mitchell E. REFF and William H. Rastetter described in

- ☒ the specification filed herewith  
☐ Application No. \_\_\_\_\_, filed \_\_\_\_\_  
☐ Patent No. \_\_\_\_\_, issued \_\_\_\_\_.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern, or organization having rights to the invention is listed below,\* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27.)

NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_  
[ ] individual [ ] small business concern [ ] nonprofit organization

NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_  
[ ] individual [ ] small business concern [ ] nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee and any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b).)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Kenneth J. Woolcott  
TITLE OF PERSON OTHER THAN OWNER Vice President, General Counsel and Licensing Executive  
ADDRESS OF PERSON SIGNING 11011 Torreyana Road, San Diego, CA 92121

SIGNATURE \_\_\_\_\_ DATE \_\_\_\_\_